RESPONSE OF RHIZOBACTERIAL COMMUNITY TO AGRONOMIC PRACTICES IN CHICKPEA FIELD, AND ITS EFFECTS ON PULSE-CEREAL ROTATION SYSTEM

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ABSTRACT

In chickpea production located in prairie ecozone, late-maturing genotype combined with current fungicide application practices may negatively impact soil bacteria composition and their biological functions, which may further influence the structure and activity of the rhizobacterial community of a following durum wheat crop.

In field experiment I, three fungicide treatments and one control were used to test the influence of toxic chemical compounds on chickpea rhizobacterial community. Results show that different fungicide application strategies negatively affect the composition of rhizobacterial communities. The richness of the bacterial communities significantly changed between the two experimental years, indicating that environmental factors further influence the effects of fungicide application on rhizobacterial growth.

In field experiment II, one yellow pea and three chickpea cultivars were used to test the impact of different pulse genotypes on rhizobacterial communities. Results demonstrate that pulse crops selectively influence the composition of their associated rhizobacterial communities. It was confirmed by a greenhouse bioassay, as wheat showed higher biomass production after yellow pea and CDC Luna chickpea than after CDC Vanguard and CDC Frontier.

In a two-year crop rotation field trial conducted in the same field as experiment II, durum wheat was planted after pulses to test the effect of different previous pulse crops on the root endophytic bacterial community in a following durum wheat crop. Results indicate that the richness and composition of durum wheat endophytic bacterial communities may change with pulse crops, and these changes correlated with wheat yield, under field conditions. The better yield of wheat after pulses may be related to the

release of hydrogen gas by their root nodules, which augment the abundance of H_2-oxidizing rhizobacteria. The latter show an ability to promote plant growth under tested *in vitro* conditions.

Finally, this microbial study reveals that the cropping practices influence the diversity and composition of chickpea rhizobacterial community. Shifts in the functional groups of soil bacteria may affect the overall microbial activities with important ecological consequences for each particular cropping system. Therefore, agronomic decisions reinforcing the beneficial microbial communities and its biological functions could improve the soil quality and efficiency of Prairie cropping systems.

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DEDICATION

I dedicate my thesis to my

Parents

ShudongYang and Li Yang

whose efforts and ever willing support have made this dream come ture.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
CDC	Crop development centre
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel elecrophoresis
DNA	Deoxyribonucleic acid
FAMEs	Fatty acid methyl esters
GA	Gibberellins
GHG	Green house gas
H_2	Hydrogen
IAA	Indoleacetic acid
LB	Luria-bertani
LSD	Least significant difference
MID	Multiplex identifiers
MRPP	MultiResponse permutation procedure
MSA	Mineral salt agar
N	Nitrogen
N_2	Nitrogen
N_2O	Nitrous oxide

NADH	Nicotinamide adenine dinucleotide hydride
NCBI	National center for biotechnology information
NH_4NO_3	Ammonium nitrate
OTUs	Operational taxonomic units
P	Phosphorous
PBI	Plant biotechnology institute
PCR	Polymerase chain reaction
PGPR	Plant-growth-promoting rhizobacteria
RCBD	Randomized complete block design
RDA	Redundancy analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SH	Sulfhydryl
TE	Tris-ethylenediaminetetraacetic acid
TEFAP	Tag-encoded 454 GS FLX amplicon pyrosequencing
TGGE	Temperature gradient gel elecrophoresis
USEPA	United states environmental protection agency
VOCs	Volatile organic compounds
WUE	Water use efficiency

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) has shown less positive effects on the growth of a subsequent durum wheat (*Triticum turgidum* var *durum* Desf.) crop than other pulses such as pea (*Pisum sativum* L.), in cropping systems involving crop rotation (Miller et al. 2002). Until now, no results could explain entirely this observation. Fungicide use and late harvest time are typical features of chickpea production (Gan et al. 2006), but not in pea. Whether these features have important or negligible effects on soil bacterial community contributing to the rotation effect is still under controversy (Navas-Cortes et al. 1995; Pethybridge et al. 2005; Shtienberg et al. 2006). To improve knowledge on the mechanisms involved in determining the "rotation effect" mediated by microbial associates of plants, a series of hypotheses and objectives was layed out, and addressed through a series of field and controlled condition experiments:

1.1 Hypothesis

- Some active ingredients in fungicides such as chlorothalonil and azoxystrobin, which are used in chickpea production, have non-target effects on bacterial communities while killing fungal pathogens. I hypothesize that fungicide application, while increasing chickpea growth by reducing *Ascochyta rabiei* infection, changes the size, structure and diversity of the bacterial community in chickpea rhizosphere soil. In particular, the growth of some plant-growth-promoting rhizobacteria (PGPR) bacteria, such as nitrogen fixers and hydrogen oxidizers, may be affected.

- Different plant genotypes developed different root system and rhizosphere. I hypothesize that different chickpea cultivars are associated with distinct rhizobacterial communities, which are also different from the rhizobacterial community living in yellow pea rhizosphere.
- Previous research showed that chickpea offers less benefit to a following crop of durum wheat in a rotation series compared with yellow pea. Since fungicide usage and indeterminate growth habits of chickpea may influence the bacterial community in the rhizosphere, I hypothesize that yellow pea and chickpea crop select a different rhizobacterial community and affect the composition of the endophytic bacterial community in the root of a following durum wheat crop and crop yield.

1.2 Objectives

General objectives of this research project were defined in order to:

- Increase the knowledge on the effects of fungicide application on the general bacterial community and the functional bacterial groups such as nitrogen fixing bacteria of chickpea rhizosphere in field experiment I.
- Discover the relationships between the properties of the rhizobacterial communities associated with yellow pea and different chickpea cultivars, and the rotational effect of these crops, measured as the productivity of a subsequent crop of durum wheat, in field experiment II and greenhouse bioassay.

A series of field and greenhouse experiments were used to better understand the contribution of the microbial associates of plants in the “rotation effect” and on the mechanisms involved in defining this contribution. Field experiments have been

designed to produce relevant data about the effect of cropping practices on the soil system. Greenhouse experiments and laboratory experiments were also conducted to clarify the effects of experimental factors under controlled conditions and provide solid conclusions. The combination of these experimental methods allowed the identification of fungicide application and chickpea genotype impacts on chickpea rhizobacterial community and provided information on the mechanisms involved.

Cultural and molecular methods including cloning, polymerase chain reaction (PCR), denaturing gradient gel elecrophoresis (DGGE) and metagenomics method including 454 GS FLX amplicon pyrosequencing were applied in these studies. Methods in statistics appropriate for agronomic data were also designed and applied to analyse data relevant to microbial ecology and sustainable agriculture sciences.

1.3 Literature Cited

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2. LITERATURE REVIEW

2.1 Chickpea production

Chickpea (*Cicer arietinum* L.) is the third most important food legume in the world after dry bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (Ibrikci et al. 2003; Pande et al. 2005). Due to its resistance to drought, chickpea is planted in many arid and semiarid regions. India, Pakistan and Turkey produce 65%, 9.5% and 6.7% of the total annual chickpea production respectively (Millan et al. 2006). The Middle East, Australia, and Mexico are also important producing regions (Kyei-Boahen et al. 2002). As a high nutrient food crop, chickpea seeds contain approximately 30% protein, 40% carbohydrates, and 5% oil (Gil et al. 1996). Moreover, they abound in Ca, Mg, K, P, Fe, Zn, Mn, Cu, B and Ni as compared with vegetables such as spinach and cabbage (Ibrikci et al. 2003).

Chickpea is used for crop diversification in wheat-based rotation in dry areas of Canada. Chickpea can form symbioses with beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) (Tavasolee et al. 2011), nitrogen-fixing bacteria such as *Mesorhizobium ciceri* (Garg and Chandel 2011), and other plant-growth-promoting rhizobacteria (PGPR) (Khare et al. 2011; Shahzad et al. 2010). As a result, chickpea can fix N_2 from the atmosphere, use soil nutrients more efficiently, improve yield, and bring economic benefits to farmers. However, chickpea is also susceptible to some pathogens such as ascochyta blight (*Ascochyta rabiei*), which is very damaging (Atik et al. 2011). Based on previous research, up to 100% yield losses in chickpea

fields can occur in humid seasons because of ascochyta blight infection (Reddy and Singh 1990). Chogo (2003) found that infection by *A. rabiei* is an important factor influencing farmers' decisions to reduce the production of this high value crop, which was confirmed by Pande (2005).

2.2 Disease control and fungicide usage in chickpea production

In order to prevent ascochyta blight outbreaks and yield loss, different fungicides and fungicide application strategies are applied (Chang et al. 2007; Demirci et al. 2003; Wise et al. 2008). Generally, different chemicals and different chemical application times have different effects both on chickpea growth and pathogen control. Fungicides are heavily used in chickpea production which may cause environmental problems (Ghazanfar et al. 2011).

2.2.1 Fungicidal seed treatments

The use of infected seeds in chickpea fields can lead to serious disease outbreak if the weather conditions are suitable to disease spread, even if the infection rate of seeds is low (Gan et al. 2006). In order to minimize infection, fungicides should be used as seed treatments. For example, Sugha (1992) used benomy1 at a rate of 10 μg ml^{-1} to treat seeds and obtained good control. Also, tridemorph+maneb, thiabendazole, benomyl+thiram and maneb were used successfully as seed dressing fungicides (Gan et al. 2006). However, Sharafeh found that some fungicides could also inhibit seed germination (Sharafeh and Banihashemi 1992). Stunting, chlorosis, and low seed vigour are often seen after seed treatment (Kaiser and Hannan 1988). Generally, fungicides used as seed dressing inhibit fungal spore germination and mycelial growth, which benefits chickpea yield. However, even if seed dressing with fungicides is beneficial and

reduce diseases incidence, it does not eradicate plant pathogens. Therefore, other methods should also be used for better control of ascochyta blight.

2.2.2 Foliar fungicides use and their effects on rhizosphere

Foliar fungicides are commonly applied in chickpea field in addition to seed treatments. In Saskatchewan, the incidence of ascochyta blight on moderately resistant chickpea was reduced from 45% to 8% with foliar fungicides, and seed yield nearly doubled (Gan et al. 2006). Quinine-outside-inhibiting (QoI) fungicides are used widely for ascochyta blight control. Azoxystrobin (Quadris) and pyraclostrobin (Headline), the most popular QoI active ingredients, are available for use since 2002 in the USA (Wise et al. 2008). These chemicals block electron transport at the quinol-oxidizing site of the cytochrome bc1 complex in the mitochondrial respiration chain (Bartlett et al. 2002). In this way, the energy production in fungal populations is affected leading to death. Some species of fungi can synthesize single amino acid substitutions in the cytochrome b site. They have reduced sensitivity to QoI fungicide (Grasso et al. 2006). QoI fungicides have site-specific mode of action (Wise et al. 2008) that can relatively easily be overcome, and consequently, the potential for developing resistance to QoI fungicides is large in target pathogen groups, if one chemical is repeatedly used.

Another group of foliar fungicides commonly used in chickpea field contains chlorothalonil. Chlorothalonil can combine with enzymes containing a sulfhydryl (-SH) functional group in fungal cells and inhibit their activities. For example, glyceraldehyde phosphate dehydrogenase is an important enzyme of the glycolytic pathway, that catalyses glyceraldehyde-3-phosphate into 1,3-diphosphoglyceric acid. The functional group of glyceraldehyde phosphate dehydrogenase is made of cysteine residues. Cysteine has a –SH group that combines with the aldehyde group of other molecules

producing nicotinamide adenine dinucleotide hydride (NADH) and intermediate products with high energetic thioester, which synthesize into 1,3-diphosphoglyceric acid. Chlorothalonil readily combines with –SH, stopping chemical reactions of the glycolytic pathway, and destroying energy generation in microbial cells. Chlorothalonil can combine with Glutathione, which also has a –SH functional group, and inhibits detoxification activity in microbial cells too. Demirci (2003) found that chlorothalonil was the most effective fungicides among a series of chemicals, with a disease inhibition rate of 70.4%. Shtienberg (2006) also reported chlorothalonil as highly effective in disease control (88.6% to 95.3%).

2.2.3 Effects of fungicide application rate

Application rate affects pathogen control. Pethybridge (2005) found that significant reductions in colony diameter of *Phoma ligulicola* isolates only happened at the high concentration (500 ppm) of chlorothalonil application. Good inhibition effect on *A. rabiei* was also obtained at moderate concentration (130 ppm) of mancozeb, and low concentration (0.25 ppm) of pyraclostrobin (Chang et al. 2007). However, Shtienberg (2006) found that only low concentration of tested fungicides was effective in two of five experiments he studied.

These conflicting conclusions may be due to the different chemicals and concentrations applied in these studies. Besides, effects of chemical concentration can also be modified by other factors. Banniza (2011) reported that the benefit of increased fungicide applications was only expressed under high disease pressure, based on field test of several common fungicides used in chickpea production, i.e., chlorothalonil, azoxystrobin, pyraclostrobin, mancozeb and boscalid. They also reported rainfall impacts on the disease control efficiency of fungicide input, in the same study. Therefore,

optimal fungicide concentration is related to plant disease susceptibility, pathogen resistance, disease pressure, and weather conditions. It appears from all that environmental conditions and application time are also very important in disease control.

2.2.4 Effects of fungicide application time

Timely application is essential to efficient disease control. Because *A. rabiei* is an ascomycete, it can produce both ascospores and conidia. Thus, only 5~7d delay in fungicide treatment could allow the pathogen to complete many infection cycles in a cropping season (Chang et al. 2007). The first application of fungicides on the seedling and pre-flowering stages could reduce disease incidence significantly because multiplication of ascospores is reduced and risks of ascospore-induced epidemics are minimized (Banniza et al. 2011; Gan et al. 2006). Chongo (2003) reported that application of azoxystrobin at early-flowering stage could reduce final disease severity but did not affect seed yield, while applying the same fungicide at mid-flowering could reduce disease severity and increase seed yield. Multiple applications of different fungicides can effectively suppress or prevent *A. rabiei*.

The best time of fungicide application also depends on rainfall and relative humidity. Ascochyta blight could be adequately suppressed when fungicides were applied to avoid disturbance due to rain, but in time to protect plants. Results of tests using several products demonstrated that the effectiveness of fungicide treatment depends on environmental conditions (Shtienberg et al. 2006). If foliar fungicide is washed off by rain, disease will become more serious and control efficiency will be low. For example, cool and humid conditions combined with frequent rainfall increased infection rate at the seedling stage in chickpea field in southern Australia (Gan et al. 2006). Two reasons can explain this phenomenon: first, heavy rain can wash away foliar fungicide from plants,

hasten their decomposition, thus reducing the anti-fungal activity of the chemicals. Secondly, the ascospores of the pathogens could infect larger field areas because of rain splash, and produce more serious disease outbreaks.

Based on the above information, optimal fungicide application time is very important in preventing disease. Controlling disease after it happened is difficult. Multiple fungicide applications during the growing season do not eliminate the pathogen better than a single timely application. Besides, wisely chosen fungicide application time should consider climatic conditions as well.

2.3 Non-target effects of fungicide on rhizosphere soil microorganisms

2.3.1 Direct non-target effects of fungicide

In modern agriculture, fungicides are widely used to protect crops against fungal pathogens and increase yields, but they may have unexpected impacts on organisms in agroecosystems. Fungicides can directly affect non-target organisms through their effects on non-specific binding sites, as different organisms may possess identical or similar mechanisms and constituents. For example, the toxicity of carboxylic acid fungicides is derived from the ability of these chemicals to bind on DNA topoisomerase II, a common enzyme that unwinds and winds DNA to allow protein synthesis and DNA replication. This enzyme is found in fungi but also in prokaryotic cells (Sioud et al. 2009). Some glucopyranosyl antibiotic fungicides are toxic to bacteria, in which they may inhibit amino acid synthesis (Carr et al. 2005). These fungicides are also toxic to certain non-fungal eukaryotic organisms (Perez et al. 1991).

2.3.2 Indirect non-target effects of fungicide

Indirect effects on non-targeted organisms are also possible. Microorganisms are either functionally or nutritionally connected with others, and changes in any component

of a microbial community may influence the structure of the whole community. This is particularly true for plant associated microorganisms, which influence and are influenced by plant metabolic status (Černohlávková et al. 2009; Wang et al. 2004; White et al. 2010; Yen et al. 2009).

The effects and mechanisms of fungicides on microorganisms are neither fully understood nor well classified. Therefore, fungicide use may have negative impacts that are difficult to predict (Lo 2010). Table 2.1 summarizes current state of knowledge on fungicide action modes to help us anticipate their possible impacts on soil microorganisms, which is important for the establishment of a proper regulation for the use of these important agro-chemicals. Current knowledge on fungicide effects on membrane, nucleic acids and protein synthesis, signal transduction, respiration, mitosis and cell division are summarized in this table as well.

2.4 Chickpea management and effects on rhizosphere soil organisms

2.4.1 Chickpea growth period and its effects on rhizosphere soil organisms

Chickpea can be cultivated at different time during the year in different cropping areas, depending on climatic conditions. In Turkey and USA, chickpea can be planted in March and April; in the Mediterranean, seeding time can be moved up to February; in North Africa, chickpea can grow during winter, as well as in Australia (Siddique et al. 1999; Smithson et al. 1985; Yau 2005). Chickpea has an indeterminate growth habit (Anbessa et al. 2007), which leads to later harvest time in chickpea production than other pulses. Meier (2008) reported that different growth stages of a same plant can influence differently the associated rhizosphere organisms, mainly due to changes in rhizodeposition at different plant growth stages. Particularly, young roots can supply more energy to soil microorganisms as they produce more excretions than older roots

(Bowen and Rovira 1991; Lynch and Whipps 1990). Furthermore, different microbial groups have different metabolic strategies and use different organic materials (Andrews and Harris 1986), which leads to changes of the soil microbiota with changes in plant root secretions. Bacterial groups, characterized by high growth rates and high substrate requirement called r-strategist which grew better at early growth stage, as more available substrates were secreted from plant roots into soil system leading to high rhizobacterial diversity at this stage. By contrast, bacterial groups characterized by low growth rates and low substrate requirement called K-strategist, grew better at late growth stage (Andrews and Harris 1986; Zhang et al. 2011). Therefore, chickpea crops influence their associated microbial processes by modifying the nutrient resources in their habitats.

2.4.2 Effects of chickpea on rhizosphere soil organisms

Selected chickpea genotypes showed enhanced disease resistance and higher yield (Taran et al. 2009). Different plant genotypes have different effects on their associated rhizosphere microorganisms (Lupwayi and Kennedy 2007). Chickpea rhizosphere soil contains a variety of organic compounds secreted by plant roots that serve as sources of energy and nutrients for the soil macro- and microbiota (Lupwayi and Kennedy 2007). Microbial growth and population densities can be increased by the large amounts of soluble C and nutrients provided by plant roots in rhizosphere soil as compared to bulk soil (Nguyen 2003). A specific rhizospheric interaction occurring between rhizobia and legumes leads to root-nodule formation. N_2-fixing bacteria use the energy generated by legumes' photosynthesis to fix atmospheric N_2 into a plant-available form. This procedure involves bidirectional plant-bacterium signaling. The roots of chickpea release signal molecules such as flavonoids, which attract specific rhizobia to their root hairs. "Nod factors", which are lipo-chito-oligosaccharides encoded by *nod* genes in host

Table 2.1 Action modes and possible non-target effects of fungicides

Action site	Action mode	Fungicide chemical group	Common name	Non-target effects
Membrane	Lipid degradation	Aromatic hydrocarbons	Dicloran	Disruption of hydrophobic interactions (Bermúndez et al. 2008)
			Etridiazole	Retard nitrification in ammonium oxidizers (Rodgers 1986)
			Chloroneb	Affect oomycetes growth (Ingham 1985)
	Sterol degradation	Triazoles	Triadimefon	Long-term inhibiting effects on soil bacterial community (Yen et al. 2009)
	Intracellular membrane disruption	Hydrochloride	Acriflavine	Thicken peripheral cell wall of *Staphylococcus aureus* (Kawai and Yamagishi 2009)
Amino Acid and Protein synthesis	Amino acid and protein synthesis inhibitors	Glucopyranosyl antiboitic	Streptomycin	Inhibit amino acid synthesis in bacteria (Carr et al. 2005) and amphibian nerve fibers (Perez et al. 1991)
		Tetracycline antibiotic	Oxytetracycline	Also used as bactericide (Yang et al. 2009)
		Anilinopyrimidines	Cyprodinil	Toxic to aquatic invertebrates (Warming et al. 2009)
			Pyrimethanil	Inhibit algal growth (Verdisson et al. 2001)
Signal transduction	Signal transduction inhibitors	Quinoline	Quinoxyfen	Highly toxic to aquatic invertebrates (Warming et al. 2009)
		Phenylpyrroles	Fludioxonil	Toxic to algae (Verdisson et al. 2001)
		Dicarboximides	Iprodione	Affect signal transduction in bacteria (Miñambres et al. 2010)
			Vinclozolin	Adversely affect bacteria community (Banerjee and Banerjee 1991)
Respiration	NADH oxido-reductase (Complex I) inhibitors	Pyrimidinamines	Diflumetorim	Unknown
	Succinate-dehydrogenase (Complex II) inhibitors	Pyridine carboxamides	Boscalid	May affect prokaryote organisms growth (Oyedotun and Lemire 2004)
		Gxathiin carboxamides	Carboxin	
		Benzamides	Flutolanil	
	Cytochrome bc1 (Complex III) inhibitors	Methoxyacrylates	Azoxystrobin	Inhibited symbiosis of arbuscluar mycorrhiza fungi (Diedhiou et al. 2004)
		Methoxycarbamates	Pyraclostrobin	Toxic to *Trichogramma pretiosum* (Manzoni et al. 2006)
	Oxidative phosphorylation uncouplers	2,6-dinitroanilines	Fluazinam	Have potential risk to environment (van Wijngaarden et al. 2010)
	Oxidative phosphorylation inhibitors	Tri phenyl tin compounds	Triphenyltin hydride	May affect bacteria, algae, soil fauna and higher plants (Huang et al. 2002; Roessink et al. 2006)
			Triphenyltin hydrozide	
			Triphenyltin chloride	
			Triphenyltin acetate	
Mitosis and cell division	Inhibitor of spindle microtubules assembly	Methyl benzimidazole carbamate	Benomyl	May affect nitrification associated bacteria group in soil (Chen et al. 2001)
			Carbendazim	
Nucleic acids synthesis	RNA polymerase I inhibitors	Acylalanines	Metalaxyl	Affect activities of ammonifying and nitrifying bacteria in soil (Monkiedje and Spiteller 2005)
			Benalaxyl	Toxic to Eisenia fedtia (Peng et al. 2009)
		Oxazolidinones	Oxadixyl	Unknown
	DNA topoisomerase II	carboxylic acids	oxolinic acid	Also used as bactericide (Kwon et al. 2010)
	Adenosin-deaminase inhibitors	Hydroxypyrimidines	Ethirimol	Unknown

plants, induce deformation of root hair and initiate root infection and formation of nodules (Geurts et al. 2005). Through its symbiosis with N_2-fixing bacteria, chickpea could fix up to 141 kg ha^{-1} of atmospheric N (Unkovich and Pate 2000). In Saskatchewan, different chickpea cultivars fixed an average of 13 kg N_2 ha^{-1} (Thavarajah et al. 2005). Even though this number is relatively low when compared to fixed nitrogen in soybean, beans or alfalfa (Aranjuelo et al. 2008; Unkovich and Pate 2000); the net fixed nitrogen could reach 1.3 million kg in chickpea growing area of the Canadian Prairie (Lupwayi and Kennedy 2007). Also, chickpea exudes organic acids in the rhizosphere, particularly in low-P soils, which mobilizes P from pools of otherwise unavailable soil P (Veneklaas et al. 2003).

2.5 Chickpea in crop rotation

2.5.1 Roles of chickpea in crop rotation

Chickpea can be cultivated in all sorts of cropping systems and rotated with other crops (Yadav et al. 2007). Chickpea can form symbioses with N_2-fixing bacteria, PGPR or AMF, and have positive effects, such as better nitrogen nutrition and soil fertility, on following crops. For example, Ryan (2008) found that the inclusion of chickpea in a cereal-based rotation increases soil organic matter and benefit the other crops of the rotation. Chickpea can also have negative effects on a cropping system, because it is very susceptible to ascochyta blight.

A. rabiei can survive as asexual pycnidia on the debris of chickpea lying on the soil surface and cause problems in the following year (Navas-Cortes et al. 1995). Since disease breaks down quickly under warm and wet conditions, the use of non-host crops such as barley for one or two years between chickpea crops can significantly reduce the level of *A. rabiei* inoculum. However, a longer cycle of cropping rotation is needed

when the weather is dry and cold and crop residue are more persistent (Gan et al. 2006). In Swift Current, Canada, Gossen and Miller (2004) found that the severity of ascochyta blight in a susceptible chickpea cultivar was much higher after one than two intervening crops. Eighty-one percent of the field was affected after one intervening crop, compared to 4 % after two intervening crops and 5 % after three intervening crops. Even though the infection rate was much lower when more than one intervening crop was used, potential infection risk still existed after 4 years of non-host crops. It appears that the key factor determining the length of the crop rotation is the speed of residue breakdown, which is affected by weather and other environmental factors. For this reason, at least two non-host crops are needed between two crops of chickpea.

2.5.2 Effects of chickpea on associated bacterial community in rotation

The inclusion of legumes in crop rotation can break disease cycles and modify the soil microbial community (Lupwayi and Kennedy 2007). In Saskachewan, for example, Biederbeck (2005) found larger microbial population and enzyme activities in legume-wheat rotation than in fallow-wheat rotation or monocultured wheat, six years after the legume crops.

Arbuscular mycorrhizal fungi are commonly associated with pulses. These fungi enhance nutrient and water uptake by the plants and affect other fungi or bacterial groups in soil by altering nutrient status, as revealed by Johnson (1992). Besides, when a legume is used as a rotation crop before wheat or canola, these non-legume crops host more endophytic rhizobia in their root compared with monocultures. In Alberta, up to 7244 cells of endophytic rhizobia were seen in one gram of dry wheat root following uninoculated pea, but this number was ten times less in wheat monoculture (Lupwayi et al. 2004). Ascospores of pathogen such as *Cochliobolus sativus* in soil may accumulate

in monocultured wheat (Barve et al. 2003). This root rot organism can cause lesions on wheat roots system and reduce growth leading to changes in the associated microbial community.

2.5.2.1 Genotype effects of chickpea on associated bacterial community

Genotype effects of chickpea on the rhizosphere were important (Yang et al. 2012), and previous study also reported host range can influence the rhizobium isolates (Ampomah et al. 2008). The selective effects of chickpea genotype could be due to differences in root secretion between cultivars, as proposed earlier (Lupwayi and Kennedy 2007). The growth and population densities of rhizosphere bacteria can be increased by large amounts of root secretion, sloughing-off of root cap cells, and senescing root epidermis in rhizosphere soil (Nguyen 2003). The functional bacteria groups could also be influenced by differences in the symbiotic signaling physiology of different chickpea genotypes (Yang et al. 2012). Specific flavonoids produced by legumes attract specific rhizobia to their root hairs, and the rhizobia in turn, produce the "nod factors" that induce root hair infection and nodule formation (Geurts et al. 2005). There fore, differences in the signaling system of different chickpea genotypes could result in differences in the recognition pattern between the plants and associated bacterial communities in their rhizosphere.

2.5.2.2 Termination time effect of chickpea on associated bacterial community

Since compared with other pulse crops, chickpea has a late termination time, therefore, different growing habits of varied pulse crops may affect their associated rhizobacterial communities. Change in the soil microbiota following the establishment of a new crop

was shown to proceed slowly over a period of several years (Hamel et al. 2005). By contrast, the decomposition of plant residue in soil initiates within hours, indicating a rapid succession of microorganisms with increasing ability to decompose complex organic compounds (Astaraei 2008; McMahon et al. 2005). Thus, changes in the bacterial community in pulse field correlated with changes in preceding pulse crop termination time is likely attributable to temporal variation in the abundance of key microorganisms in the soil microbial pool, which are evolving under the large influence of decomposing residues.

2.6 Rhizobacteria

2.6.1 Factors influencing rhizobacteria

For decades, chemicals have been widely used for plant diseases control. Chemical pesticides control diseases and increase crop productivity, but non-selective chemicals could also suppress beneficial species along with plant pathogens. Since repeated pesticide applications also increase the resistance of some pest species, misuse of pesticides can result in more serious disease outbreak (Ferre et al., 2006; Makovitzki et al., 2007). For example, strains of *Pseudomonas syringae* pv. *syringae* resistant to copper and streptomycin (Sundin and Bender 1993), and *Erwinia amylovora* resistant to streptomycin, oxytetracycline and copper (Loper et al. 1991) were found.

Rhizosphere bacteria can be affected by soil physical properties. Firstly, soil moisture can affect bacterial growth. Bell and Raczkowski (2008) found that bacterial abundance was low in the summer time because of low soil moisture availability. Gunapala (1998) also reported that 5.4% of moisture in soil is suboptimal for bacterial growth. Secondly, soil temperature can restrict bacterial growth. High soil temperature can either directly reduce bacterial activity and microbial biomass or indirectly affect bacterial growth

through soil drying (Gunapala and Scow 1998). Besides, soil pH can impact bacterial activity in soil. Cho (2008) found that microbial biomass carbon and nitrogen could be reduced at high pH, and this impacted the microbial ecological succession in their experiments as well.

2.6.2 Rhizobacteria effects on their associated plants

Although high diverse of bacteria are living in the soil, our knowledge of these soil microorganisms is limited. Research still has a long way to go before soil bacterial biodiversity is understood. Based on current knowledge, both plant growth-promoting rhizobacteria and pathogens are living there, and exert positive, negative or even complicated interaction influencing agriculture.

There are two general types of beneficial bacteria: the symbiotic bacteria forming specialized structures such as nodules or living in plant tissues, and the free-living soil bacteria (Siddiqui 2005). The former type to which Rhizobium belong, has been studied extensively as commercial inoculants, which are applied to legume crops since several decades already. Bacteria of the second type are generally called plant growth-promoting rhizobacteria (PGPR) (Badri et al. 2009). They are species such as *Azorhizobium, Bacillus, and Pseudomonas* (Kloepper et al. 1989), which can also build mutually beneficial relationships with the roots of many crops. Some PGPR can benefit crops directly through various means such as nitrogen fixation, phosphorus solubilization, iron sequestration by siderophores, or improvement of plants ability to absorb soil water and nutrients (Lugtenberg and Kamilova 2009; Wani et al. 2007). Other species of rhizobacteria could benefit crops indirectly through antibiotic production or the induction of systemic resistance, as described by Glick (1999).

Even though most of the important phytopathogenic microorganisms are fungi, some soil bacteria are also pathogenic to plants. For example, Elyousr and Hendawy (2008) found that *Xanthomonas axonopodis pv. vesicatoria* can infect tomato seeds, and cause leaf lesions, defoliation, fruit lesions, and yield reduction.

Bacteria can multiply in one growing season and impact the next crop. For example, Govaerts (2008) found that abundance of soil bacteria increased significantly in wheat-maize rotation compared with monoculture management. Abundance of bacteria in soil could bring two different effects on crop growth. The PGPR that can fix nitrogen or solubilize phosphorus, as mentioned above, benefit the following crop because they enrich the soil. On the other hand, plant pathogens may accumulate in soil and infect subsequent susceptible crop plants (Barve et al. 2003).

2.7 Endophytic bacteria

2.7.1 Factors influencing endophytic bacteria

Endophytic bacteria were reported in many plants (Sturz et al. 2000), as plants provide diverse niches and nutrients usable by these bacteria (Rosenblueth and Martínez-Romero 2006). In a single host plant, more than one endophytic bacterial species can be found, while one endophytic bacterial species can anchor on many individual plants (Rosenblueth and Martínez-Romero 2006). Compared with rhizobacteria, endophytic bacteria would be less impacted by either biotic or abiotic stresses than soil bacteria, due to the protection provided by host plant tissues (Hallmann et al. 1997). However, changes in habitats can influence endophytic communities (Seghers et al. 2004). Endophytic bacteria have very close relationships with their host plants, and the physiological status of their host plants could remarkably influence endophytic bacteria growth. Kuklinsky (2004) found that different plant genotypes,

growth stages, and tissues can influence colonization of endophytic bacteria due to changes of binding sites on root surface and nutrient availability for bacterial growth. The influence of soil type on wheat-associated endophytic bacteria was also found (Conn and Franco 2004). Environmental changes may influence endophytic bacteria by impacting plant growth. Furthermore, endophytic bacteria are sensitive to the chemicals used in cultivated fields. Glyphosphate, a widely used herbicide in agricultural systems, can modify the composition of the endophyte community in soybean, and the plant-growth-promoting and biological control activities of these microorganisms (Kuklinsky-Sobral et al. 2005).

2.7.2 Effects of endophytic bacteria on their host plants

Although endophytic bacteria have lower population densities than rhizobacteria (Rosenblueth et al. 2004), their benefits to the host plants are considerable. Endophytic bacteria can improve nutrient availabilities to their host plants and support their growth. For example, inoculation of wheat with *Klebsiella pneumoniae* can relieve nitrogen deficiency symptoms and increase total nitrogen in the plant (Iniguez et al. 2004). Such research work extends the research of biological nitrogen fixation (BNF) to non-leguminous plants. Phosphate, another very important chemical element supporting plant growth, can be solubilized by bacterial secretions and used by the host plants (Sessitsch et al. 2002).

Endophytic bacteria can produce phytohormones stimulating their host plants' growth. Abscisic acid (ABA), indoleacetic acid (IAA) and gibberellins (GA), the common phytohormones, were secreted by, and detected in endophytic bacteria (Piccoli et al. 2011), which emphasize the role of endophytic bacteria in their host plants growth, especially under adverse environmental conditions. Inhibition of phytopathogens is

another beneficial effect of endophytic bacteria on their host plants. For example, the endophytic bacteria *Serratia plymuthica* isolated from the root of potato, showed a very strong antifungal activity protecting the host plant (Berg et al. 2005). In wheat, endophytic bacteria belonging to *Actinobacteria* also inhibited the growth of the pathogenic fungus *Gaeumannomyces graminis* (Coombs et al. 2004).

In crop rotations, allelopathic effects caused by endohytic bacteria can influence the growth of other crops in rotation series. Red clover (*Trifolium pratense* L.) inhibited maize emergence and retarded maize growth, due to allelopathic chemicals secreted by endophytic bacteria competing with other species for limited nutrients and ecological niches (Sturz and Christie 1996). Some endophytic bacteria such as *Microbacterium esteraromaticum* and *Tsukamurella paurometabolum* can suppress root-lesion nematode proliferation, and this inhibition is transferable to other crops in the cropping system (Sturz and Kimpinski 2004).

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3. Preface

The following chapter has been accepted by Applied Soil Ecology (C. Yang, C. Hamel, Y. Gan, V. Vujanovic. 2012. Tag-encoded pyrosequencing analysis of the effects of fungicide application and plant genotype on rhizobacterial communities. Applied Soil Ecology. In Press), and another paper based on results of VOCs has been accepted by Phytochemistry (A.F. Cruz, C. Hamel, C. Yang, T. Mastubara, Y. Gan, A.K. Singh, K. Kuwada and T. Ishii. 2012. Phytochemicals to suppress Fusarium head blight in wheat-chickpea rotation. Phytochemistry. In Press). The work reported here is a demonstration of the fungicide application effects on rhizobacterial community in chickpea field. By using new molecular technology 454 GS FLX amplicon pyrosequencing, this study reports the composition changes of chickpea rhizobacterial communities among different fungicide application strategies. On this basis, it is important to recognize the impact of fungicide application practices on rhizobacterial community and their potential functionalities in chickpea production.

C. Hamel and V. Vujanovic co-supervised this work. K. Hanson and C. Mcdonald provided assistance with field work. All co-authors reviewed the manuscript. I planned the experiment, processed and analysed samples, submitted DNA samples for determination at Génome Québec, Montréal, Canada. I interpreted the data and prepared the manuscript for publication.

3. TAG-ENCODED PYROSEQUENCING ANALYSIS OF THE BACTERIAL COMMUNITIES OF CHICKPEA RHIZOSPHERE AS AFFECTED BY FUNGICIDE APPLICATION

3.1 Abstract

Using polymerase chain reaction (PCR) with bar-coded primers and 454 GS FLX amplicon pyrosequencing, the present study demonstrates that fungicide application on field chickpea crops has non-targeted consequences on the rhizobacterial community. Split-plot field experiments were conducted to determine the effects of four foliar fungicide application programs on two chickpea cultivars. The bacterial richness, reflected by the number of operational taxonomic units (OTUs), Chao 1 and ACE richness estimators, only differed between two experimental years, but not among fungicide treatments, nor chickpea genotypes. However, different intensities of fungicide application significantly affected the composition of the bacterial communities compared to untreated plots, which also differed with chickpea genotype, as revealed by heat map analysis and correspondence analysis. Based on these results, it was concluded that foliar fungicide applications can impact the rhizobacterial community, and these effects can be modified by plant genotype.

3.2 Introduction

High nutritional value for humans (Gil et al. 1996; Ibrikci et al. 2003) and adaptation to arid environments (Gan et al. 2009) have made chickpea (*Cicer arietinum L.*) the third

most important leguminous crop worldwide (Ibrikci et al. 2003; Pande et al. 2005), despite its sensitivity to ascochyta blight, a devastating fungal disease (Gan et al. 2006). The fungicides intensively used in the production of chickpea crops to control ascochyta blight may have undesirable effects on other soil organisms (Gaind et al. 2007).

Bacteria are the most abundant and diverse group of soil organisms (Gans et al. 2005). They mediate many soil processes (Garbeva et al. 2004; Van Elsas et al. 2002) and play a pivotal role in maintaining soil properties for sustainable food production. Researchers have examined the effects of land use (Acosta-Martínez et al. 2008), soil pH gradient (Rousk et al. 2010), heavy metal pollution (Vishnivetskaya et al. 2011), hydrocarbon contamination (Singleton et al. 2011), and tillage and crop rotations (Yin et al. 2010) on soil bacterial communities, whereas other research examined the relationship between bacterial communities and soil quality. Some of the soil bacterial communities have important ecological functionalities involving soil formation, nutrient cycling and greenhouse gas emission (Gan et al. 2011; Nannipieri et al. 2008). Despite these key roles of bacteria in soil processes, the influence of crop production on the soil bacterial community remains largely unknown (Lo 2010).

Research on soil bacteria based on culture-dependent methods draws a partial picture of the soil microbial community (Nannipieri et al. 2008; Nautiyal et al. 2008). Physiological methods, such as fatty acid methyl esters (FAME) profiling, provide information on both culturable and unculturable soil microorganisms, which help improve our understanding of the soil microbial community (Bååth and Anderson 2003). FAME profiling can provide information on the structure of the entire soil microbial community, but it cannot detect effects at a fine level of resolution and often has to be complemented with other methods. Cloning, polymerase chain reaction (PCR),

denaturing gradient gel elecrophoresis (DGGE) and temperature gradient gel elecrophoresis (TGGE) have been used to describe the diversity of soil bacterial communities at a fine level (Bürgmann et al. 2005; Bürgmann et al. 2004). However, these technologies lack sensitivity and often underestimate diversity when the number of species is high or when dominant taxa prevail in rhizosphere samples. Recently, a new molecular technology called tag-encoded 454 FLX amplicon pyrosequencing (TEFAP) has been used successfully in the study of soil microbial ecology and, in many cases, was superior to traditional methods (Margulies et al. 2005). Therefore, I used 454 pyrosequencing as the core method in the present study to determine the effects of fungicide application on the rhizobacterial community of field-grown chickpea.

3.3 Materials and methods

3.3.1 Experimental design and site description

The experiment was conducted at the South Farm of the Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan, Canada (50º25'N, 107º44'W), in 2008 and 2009. Two factors were arranged in a split-plot design with four replicates. Two chickpea cultivars CDC Luna and CDC Vanguard, were randomized within main plots and four fungicide treatments - three application strategies plus a no-fungicide control (Rochester et al. 2001) were randomized within subplots. The fungicides Bravo® (Syngenta Crop Protection Canada Inc., Guelph, ON., with chlorothalonil as active ingredient) and Headline® Duo (BASF Canada Inc., Mississauga, ON., with pyraclostrobin and boscalid as active ingredients), two commercial fungicidal products commonly used for disease control in chickpea, were used in different application programs (Table 3.1).

Table 3.1 Timing of application and type of fungicide making up the foliar fungicide treatments used in the experiment.

Treatment	Chickpea Growth Stage				
	Seedling	Vegetative	Early-flower	Mid-flower	Podding
Control (C)	/	/	/	/	/
I	Headline® Duo	/	Headline® Duo	/	/
II	Headline® Duo	Bravo®	Headline® Duo	/	/
III	Headline® Duo	Bravo®	Headline® Duo	Bravo®	Bravo®

Note: Bravo® was applied at a rate of 1.0 kg a.i. ha^{-1} chlorothalonil; Headline® Duo was applied at a rate of 100 g a.i. ha^{-1} pyraclostrobin and 240 g a.i. ha^{-1} boscalid.

In both years, the soil (Orthic Brown Chernozem) was with organic C content of 20 g kg^{-1}, pH ($CaCl_2$) of 6.5 in the top 0-15 cm depth, and a silt loam texture with 28% sand, 49% silt and 23% clay. It contained 3.6 kg ha^{-1} N, 21.8 kg ha^{-1} P and 283 kg ha^{-1} K in 2008; and 3.1 kg ha^{-1} N, 12.6 kg ha^{-1} P and 210 kg ha^{-1} K in 2009. No legumes had been grown on the land for at least 5 years before the experiment was initiated. Average monthly precipitations during the growing season (1 April to 30 September) were recorded by a meteorological station located about 300 m from the experimental sites. In particular, average monthly precipitations during the growing season (1 April to 30 September) were 59.3 mm in 2008 and 35.6 mm in 2009.

3.3.2 Soil and plant sampling

Rhizosphere soil samples were taken at harvest time of chickpea crop in September. The first 1 cm of surface soil was removed to eliminate plant debris, and five chickpea plants were dug from each plot. After gently shaking off the bulk soil, plant samples with rhizosphere soil from each plot were pooled together and labelled. Samples were put on ice and taken back to the lab. In the lab, rhizosphere soil was brushed off from chickpea roots, then put through 2 mm sieves. Samples were stored in a plastic bag at -20 °C for further analysis. Chickpea plants collected from each plot were washed with tap water to remove surface dirt, separated into roots and leaves, and kept in separate sealed plastic bags for further analysis.

3.3.3 Soil DNA extraction and PCR for bacterial tag-encoded 454 GS FLX amplicon pyrosequencing

Raw DNA was extracted from soils using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), as recommended by the manufacturer, and diluted 20 times. The DNA extracts were subjected to PCR using 16S rDNA-targeting

primers 968f / 1401b amplifying an approximately 450bp fragment of the bacterial universal gene. The primers had a 454 Life Science's A or B sequencing adaptor fused to the 5' end of the forward and reverse primers (Table 3.2). One of 16 unique multiplex identifiers (MID) was added between the A sequencing adaptor and the forward primer. Platinum® PCR SuperMix (Cat. No. 11306-016, Invitrogen™) was the PCR reaction mix. Thermal cycling was conducted in an Veriti™ 96-well fast Thermal Cycler (Applied Biosystems, CA, USA) under the following conditions: 4 min initial denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 56 °C and 1 min elongation at 72 °C; and a 15 min final elongation at 72 °C. All PCR products were purified through gel electrophoresis. Briefly, PCR products were run in 1% (w / v) agarose gel under 65 V for 1 h, and the bands migrating at the target location were excised with a sterilized scalpel blade. Excised bands were placed in sterile centrifuge tubes with 30 µl TE (Tris-Ethylenediaminetetraacetic acid) buffer (1× dilution), vortexed for 1 min and left over night at 4 °C. The purified PCR products were transferred into new tubes and their concentration was measured using a Nano Drop-1000 spectrophotometer (Thermo Scientific®, Wilmington, USA). The concentration of each sample was adjusted to 20 ng DNA • μl^{-1}. Four pools of 16 samples with different MIDs (Multiplex Identifiers) were prepared and submitted to Génome Québec (Montréal, Canada) for sequencing.

3.3.4 Analysis of plants' volatile organic compounds (VOCs)

Five chickpea plants and roots were collected with a shovel on 3 September (just after the last fungicide application) in control and fungicide treatment III plots. Shoots were separated from roots and roots were washed under running tap water. Leaves and roots were separately ground with a mortar and pestle. Two sets of 2-g subsamples of each

Table 3.2 Primers used to amplify bacterial sequences from soil DNA for pyrosequencing analysis to verify population differences

Name		Primer sequence (5'~3')
Forward Primer	Lib-L PrimerA1-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACGAGTGCGTAACGCGAAGAACCTTAC
	Lib-L PrimerA2-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACGCTCGACAAACGCGAAGAACCTTAC
	Lib-L PrimerA3-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGACGCACTCAACGCGAAGAACCTTAC
	Lib-L PrimerA4-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGCACTGTAGAACGCGAAGAACCTTAC
	Lib-L PrimerA5-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATCAGACACGAACGCGAAGAACCTTAC
	Lib-L PrimerA6-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATATCGCGAGAACGCGAAGAACCTTAC
	Lib-L PrimerA7-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGTGTCTCTAAACGCGAAGAACCTTAC
	Lib-L PrimerA8-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CTCGCGTGTCAACGCGAAGAACCTTAC
	Lib-L PrimerA10-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCTCTATGCGAACGCGAAGAACCTTAC
	Lib-L PrimerA11-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TGATACGTCTAACGCGAAGAACCTTAC
	Lib-L PrimerA13-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CATAGTAGTGAACGCGAAGAACCTTAC
	Lib-L PrimerA14-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGAGAGATACAACGCGAAGAACCTTAC
	Lib-L PrimerA15-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**ATACGACGTAAACGCGAAGAACCTTAC
	Lib-L PrimerA16-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCACGTACTAAACGCGAAGAACCTTAC
	Lib-L PrimerA17-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGTCTAGTACAACGCGAAGAACCTTAC
	Lib-L PrimerA18-F	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCTACGTAGCAACGCGAAGAACCTTAC
Reverse Primer	Lib-L PrimerB-R	**CCTATCCCCTGTGTGCCTTGGCAGTCTCAG**CGGTGTGTACAAGACCCGGGAACG

Note: Adaptor A for forward primers and adaptor B for reverse primer were shown in bold letters; 16 unique multiplex identifiers (MID) connected with forward primers were shown with underline.

type of plant tissue (leaves and roots) were collected for the identification and quantification of the VOCs they contained following the protocol produced by the research laboratory of Kyoto Prefectural University (Cruz et al. 2012). In particular, tissues were mixed with 10 mL distilled water and centrifuged at 3,000 x g for 5 min. The supernatant was collected and diluted 5 times with distilled water. Then, 12 mL of this solution was placed in vials with septum fitted caps and 3.6 g NaCl was added. The samples were then kept in the freezer until analysis. The VOCs in the gas phase of the headspace in the vials were identified by GC-MS (Hewlett Packard HP5973, USA). The injector temperature was 85 °C, and injection, split less mode was used. The capillary column was HP-WAX bonded polyethylene glycol (60 m × 0.25 mm ID, 0.5 μm). The initial oven temperature of 40 °C was maintained for 15 min, slowly increased to 100 °C at a rate of 1 °C min^{-1}, increased to 240 °C at 2 °C min^{-1}, and maintained at 240 °C for 5 min, in order to identify more VOCs. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} and an inlet pressure of 10 psi.

The 2-g subsamples of the second set were extracted by soaking in 2 mL of methanol (MeOH) at 4 °C until gas chromatography (GC) analysis. The MeOH extracts were analyzed using a GC (GL Science GC353B, Tokyo) equipped with a column TC-FFAP (30 m × 0.25 mm ID, 0.25 μm). FID injector and detector temperature was 180 ºC. The oven temperature program was as follows: 60 ºC for 5 min, increased to 120 ºC at 4 ºC min^{-1}, and then 120 ºC was maintained for 10 min. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} and inlet pressure of 10 psi. The amounts of VOCs were quantified by comparison with known amounts of chemicals standards (1-penten-3-ol, trans-2-hexenal, 1-hexanol, cis-3-hexen-1-ol, trans-2-hexen-1-ol) purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.3.5 Statistical analysis

All DNA sequences were edited to remove primers, MIDs, and adaptor sequences, using Mothur V.1.15.0 (Schloss et al. 2009). Sequences sharing 97% similarity were grouped as one OTUs (Wu et al. 2010), and classified into their respective phyla by comparison of representative sequences to sequences from The Silva database (http://www.arb-silva.de/). The edited sequence data were presented by rarefaction analysis (Nacke et al. 2011), heat map analysis (Vishnivetskaya et al. 2011), and classification of dominant phyla (Qian et al. 2011) based on identified OTUs (Operational Taxonomic Units), using Mothur (http://www.mothur.org/wiki/Thetayc). Heat maps were drawn based on Yu & Clayton theta similarity coefficient, the significance of differences in bacterial community composition between treatments was tested by parsimony analysis in Mothur (Pei et al. 2004). Chao 1 and ACE richness estimators were calculated in Mothur as well. The effects of fungicide application, chickpea genotype and year on the number of identified OTUs, Chao 1 and ACE richness estimators, and on VOC concentrations were tested by ANOVA, and the significance of differences between treatment means tested with Fisher-LSD test at α = 5% level, in SYSTAT 12.0 (http://www.systat.com/Default.aspx). Correspondence analysis was also conducted in SYSTAT 12.0, to assess the relationship between experimental treatments and the frequency of the detected OTUs classified into bacterial phyla.

3.4 Results

3.4.1 Influence of fungicide on chickpea rhizobacterial community

Rarefaction curves showed very similar patterns of chickpea rhizobacterial communities among different treatments, in both experimental years (Figure 3.1),

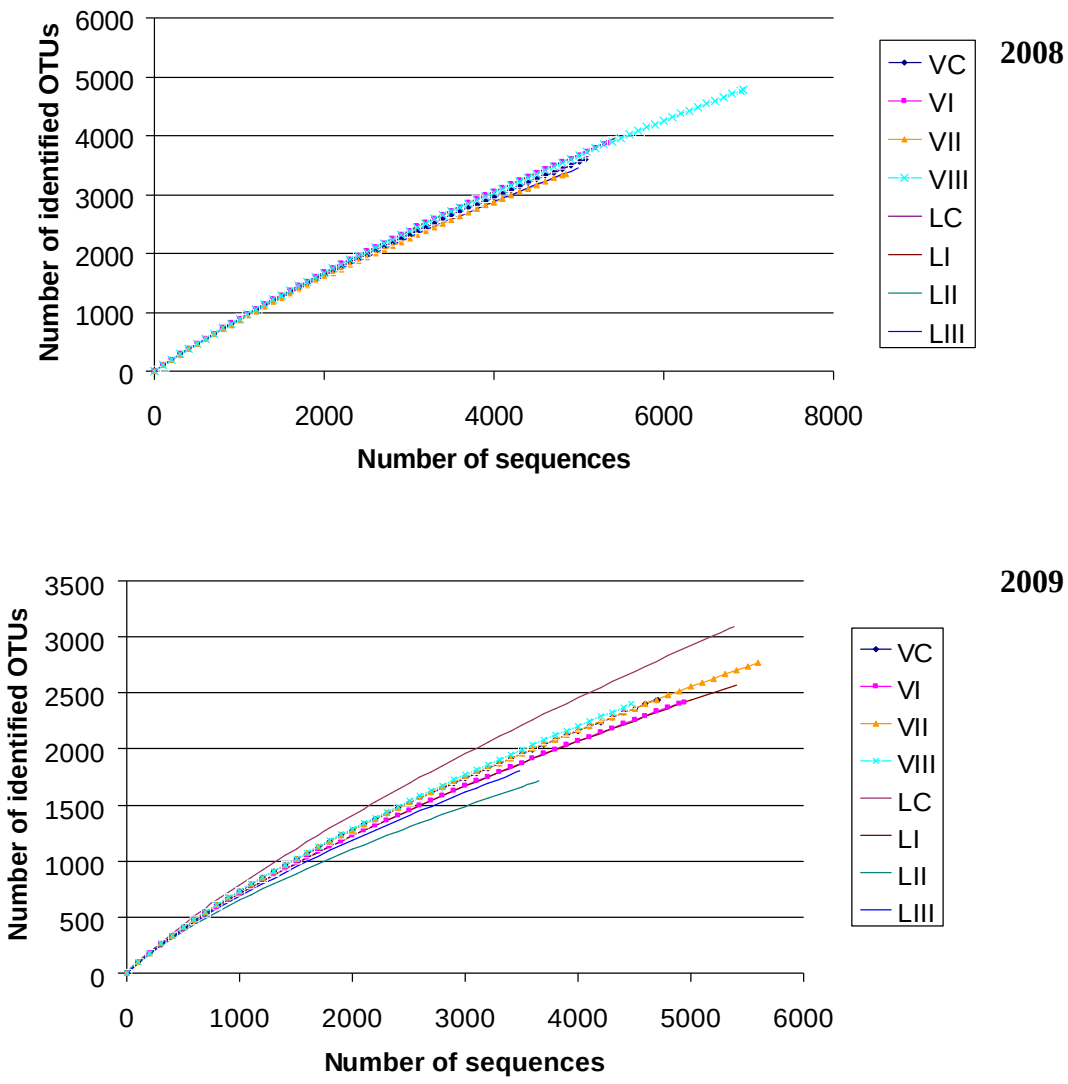

Figure 3.1 Rarefaction curves indicating the number of total identified operational taxonomic units (OTUs) at a genetic distance of 97% similarity in different treatments of fungicide and chickpea genotypes in 2008 and 2009. VC: CDC Vanguard Control; VI: CDC Vanguard treatment I; VII: CDC Vanguard treatment II; VIII: CDC Vanguard treatment III; LC: CDC Luna Control; LI: CDC Luna treatment I; LII: CDC Luna treatment II; LIII: CDC Luna treatment III.

Table 3.3 ANOVA for bacterial richness in rhizosphere soils from two cultivars of chickpea with different fungicide treatments.

Factors		Chao 1		ACE	
		Mean	*P* value	Mean	*P* value
Cultivar	CDC Luna	2596	ns	4838	ns
	CDC Vanguard	2755		5276	
Fungicide	Control	2661	ns	4894	ns
	I	2888		5470	
	II	2484		4869	
	III	2666		4994	
Year	2008	3198 a	<0.001	6089 a	<0.001
	2009	2152 b		4025 b	

Note: ns means not significantly different at $P < 0.05$; N=64

Table 3.4 Concentrations (nl g^{-1} fresh weight) of volatile organic compounds in the leafs and roots of chickpea tissues as influenced by genotype and fungicide application in 2008.

	Leaf			Root
Treatment	1-Penten-3-ol	Cis-3-hexen-1-ol	Trans-2-hexen-1-ol	1-Penten-3-ol
LC	164 b[*]	111 b	85.3 a	142 b
VC	408 a	261 a	68.0 a	306 a
LIII	59 c	67 c	nd[§]	68 c
VIII	80 c	40 c	33.1 b	65 c
	$P < 0.0001$	$P < 0.0001$	$P = 0.0344$	$P < 0.0001$

Note: [*]Means ($n = 4$) followed by different letter are significantly different at $P < 0.05$.
[§]nd, not detected.

indicating the total identified OTUs of chickpea rhizobacteria did not vary much with fungicide treatment. This was also confirmed by ANOVA (Table 3.3). However, ANOVA of VOCs showed a significant difference (Table 3.4) between with the fungicide treated and non-treated chickpea plants in 2008, suggesting that fungicides modified chickpea plant physiology, rather than the directly effects of fungicide, is the reason to explain rhizobacterial community changes with fungicide applications.

Among the 11 identified bacterial phyla, the phylum Acidobacteria comprised 22% - 33% of total identified OTUs, depending on treatments (Figure 3.2). Proteobacteria, the second largest phylum with 19%- 23% of the OTUs, was only slightly more abundant than the Actinobacteria, which accounted for 19% - 22% of the identified OTUs. The Gemmatimonadetes made up 4% - 5%, and the Verrucomicrobia, 3% - 4% of the OTUs identified. The other six identified bacterial phyla, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes and Nitrospirae, represented less than 1.5% of the identified OTUs. The influence of fungicide on these bacterial phyla was detected by correspondence analysis showing significant selective effects of fungicide treatments on associated identified bacterial phyla of both years (Figure 3.3 and Figure 3.4).

The effects of fungicide treatments on the composition of the bacterial community were shown in the heat map, where the Yue & Clayton Theta similarity coefficient between control plots and fungicide treated plots were low (Figure 3.5), especially in 2009. Parsimony analysis also showed significant differences in bacterial composition among fungicide treatments ($P < 0.001$), confirming that fungicide influenced the composition of chickpea rhizobacterial communities.

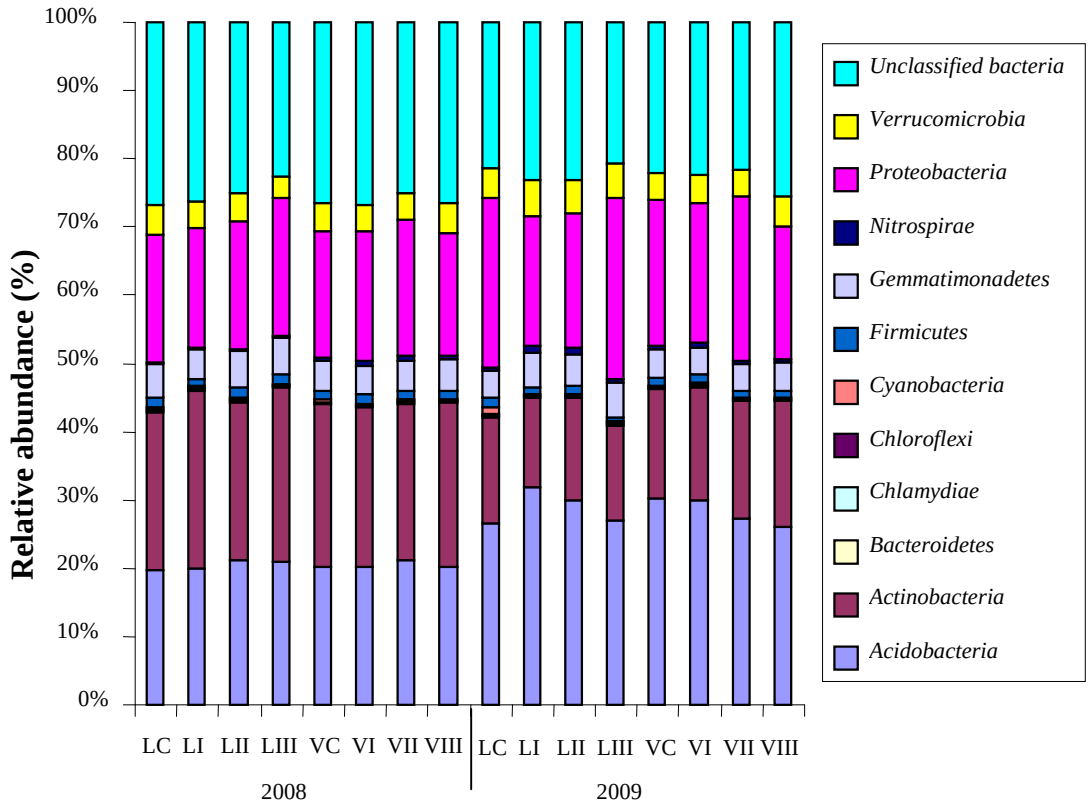

Figure 3.2 Classification of identified rhizobacterial phylum showing relative abundances of phylogenetic groups in rhizosphere soils derived from the different fungicide treatments and chickpea genotypes.

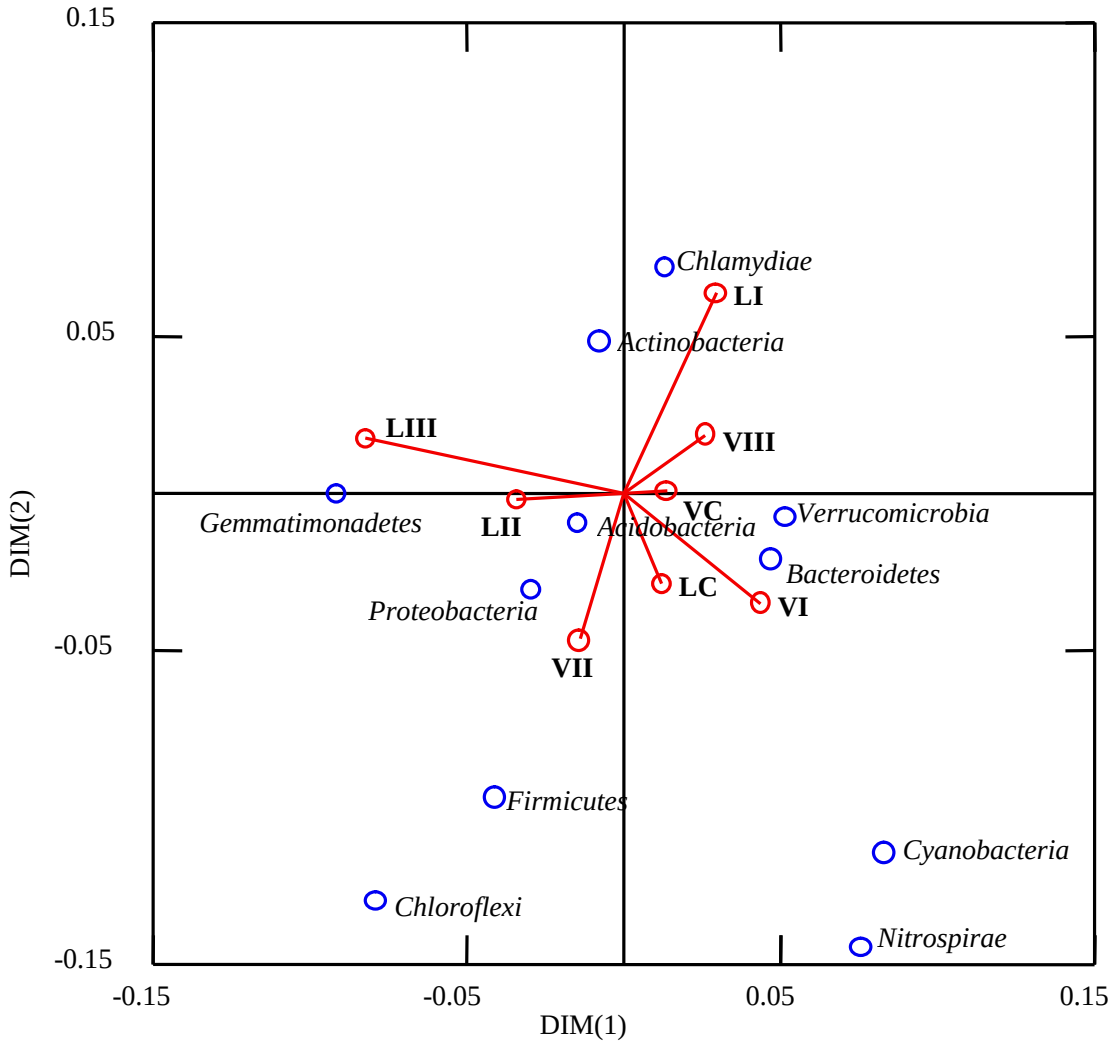

Figure 3.3 Correspondence analysis of relationships between fungicide treatments and identified bacterial phylum in the rhizosphere of both chickpea cultivars in 2008. (P = 0.011, N = 32).

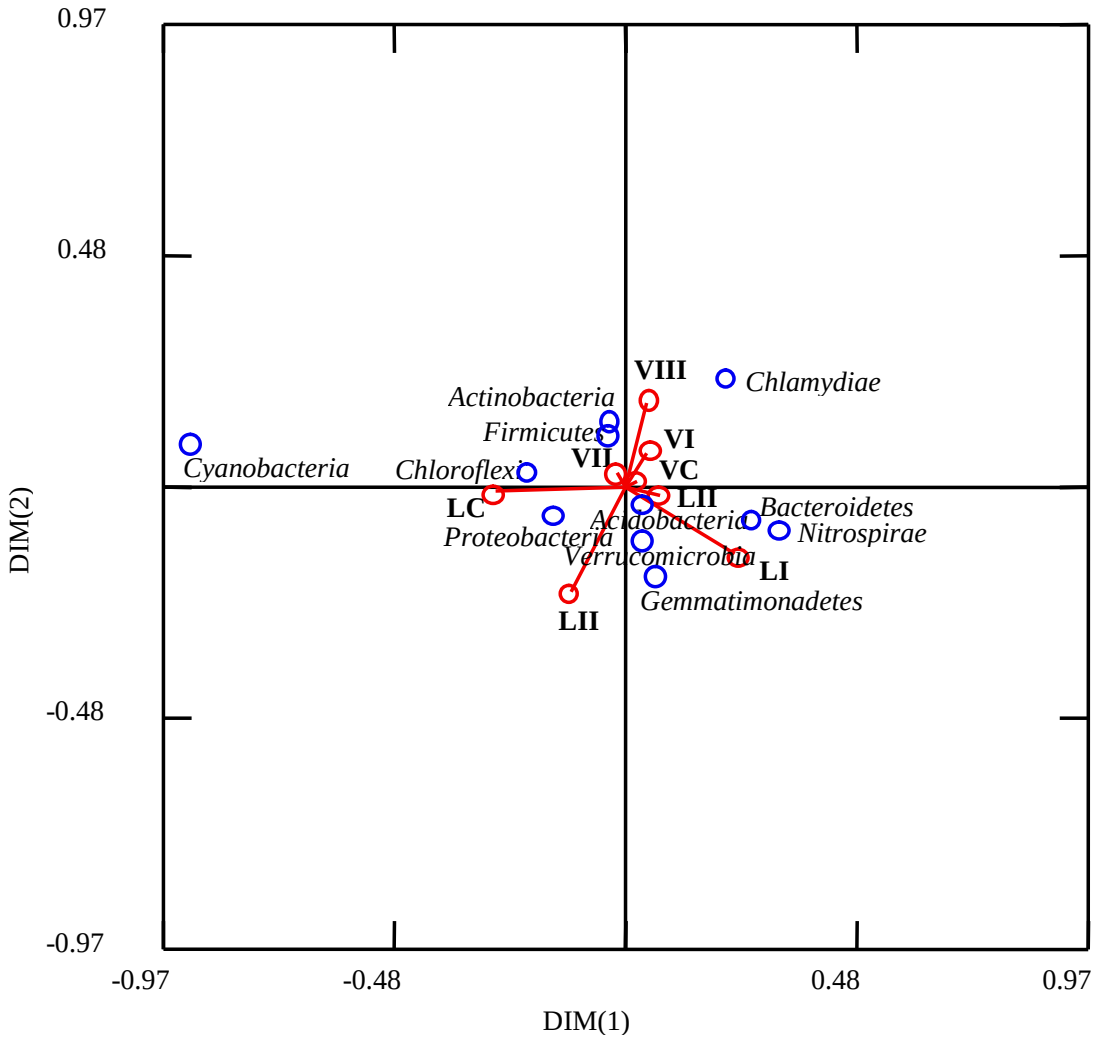

Figure 3.4 Correspondence analysis of relationships between fungicide treatments and identified bacterial phylum in the rhizosphere of both chickpea cultivars in 2009. (P < 0.001, N = 32).

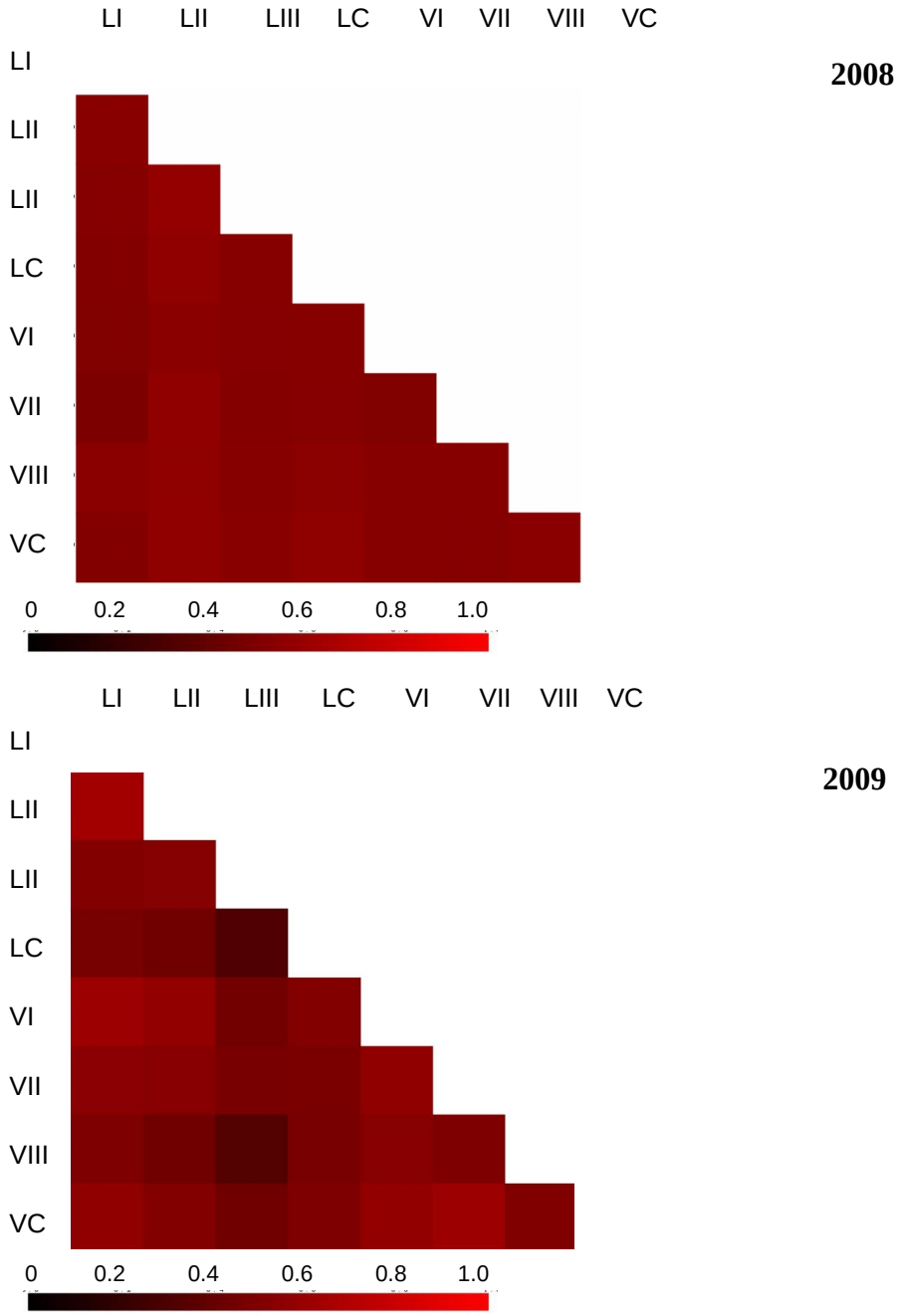

Figure 3.5 Heat map representation of fungicide and genotype effects on the composition of the rhizobacterial community of chickpea crops in 2008 and 2009. The shade of the red colour represents the degree of Yu & Clayton theta similarity in bacterial communities. As shown in the scale, black color means 0% similarity between tested rhizobacterial communities, and light red color means 100% similarity between tested rhizobacterial communities.

3.4.2 Influence of chickpea genotypes on rhizobacterial community

No significant difference in rhizobacterial richness was found between the two chickpea cultivars (Table 3.3). However, CDC Luna and CDC Vanguard had different rhizobacterial community composition, according to the Yue & Clayton Theta similarity coefficient (Figure 3.5) and parsimony analysis ($P < 0.001$). This effect of plant genotype on rhizobacterial community composition was modified by the fungicide treatments. Under fungicide treatment III, the relative abundance of Proteobacteria increased in CDC Luna plots but decreased in CDC Vanguard plots, as compared to controls, and the Acidobacteria in CDC Luna plots were more sensitive to the fungicide treatments than in CDC Vanguard plots, especially in 2009 (Figure 3.2).

3.4.3 Environmental effects on chickpea rhizobacterial community

Significant year effects on the bacterial communities were found (Table 3.3) where the relative abundances of Acidobacteria, Actinobacteria and Proteobacteria between the two experimental years were different ($P < 0.001$). Both Chao 1 and ACE richness estimators were significantly lower in 2009 than in 2008 ($P<0.001$, Table 3.3), which could be due to differences in precipitation during the growing season of these two years.

3.5 Discussion

Foliar fungicide application against ascochyta blight is commonly practiced in chickpea production worldwide (Chang et al. 2007; Demirci et al. 2003; Wise et al. 2008). Fungicide application may have negative impacts on soil bacteria communities that are difficult to predict (Lo 2010) in the very complex soil habitats. Under control conditions, it was reported that fungicides can influence bacterial growth by changing their membrane structure (Yen et al. 2009), nucleic acids synthesis (Kwon et al. 2010), protein synthesis (Carr et al. 2005), signal transduction (Miñambres et al. 2010),

respiration (Roessink et al. 2006), mitosis and cell division (Chen et al. 2001). Fungicide can directly affect non-target organisms through their effects on non-specific binding sites, as different organisms may possess identical or similar mechanisms and constituents. For example, carboxylic acid-based fungicides bind with DNA topoisomerase II, a common enzyme that unwinds and winds DNA to allow protein synthesis and DNA replication. This enzyme is found in fungi but also in prokaryotes (Sioud et al. 2009). Glucopyranosyl antibiotic fungicides may negatively affect bacterial growth by inhibiting amino acid synthesis (Carr et al. 2005).

Indirect effects of fungicides on non-targeted organisms are always possible, as microorganisms can be either functionally or nutritionally connected with each others. Fungicide input changing any targeted component of a microbial community may influence non-target microorganisms and the structure of the whole community (White et al. 2010), their growth (Wang et al. 2004; Yen et al. 2009), and their associated biological functionalities (Černohlávková et al. 2009).

Twelve bacterial phyla and thousands of OTUs were identified from the chickpea rhizosphere soil using the pyrosequencing approach. Proteobacteria, one of the dominant bacterial phylum detected in the chickpea rhizosphere in this study, is well known for its N_2-fixing members in pulses (Bürgmann et al. 2005; Lindström et al. 2010), which may be affected by Boscalid (APVMA 2004). I found fungicide use changed the composition of rhizobacterial community. Among the most abundant bacterial phyla identified in this study, members of the Actinobacteria were reported to induce plant systemic acquired resistance (SAR) or activate the plant jasmonate / ethylene pathways (Conn et al., 2008), leading to phytoalexin and VOCs production (Arimura et al., 2009) and thus favoring the maintenance of a healthy plant-associated biodiversity (Conn et al., 2008).

However, toxic biocide inputs may not always cause noticeable changes in total diversity of the microbiota in soil (Thirup et al. 2001). In this study, total microbial richness under fungicide treatments were not different, as different soil bacterial groups have different metabolisms. Therefore, application of particular fungicide may inhibit the growth of some bacterial groups while stimulating others (Huang et al. 2010), leading to similar richness levels in different bacterial communities.

The composition changes of rhizobacterial communities among fungicide treatments were modified by chickpea genotypes. Plant effects on their associated rhizobacterial growth were reported, as growth and population densities of rhizobacteria can be stimulated by some root secretions, sloughing-off of root cap cells, and senescencing root epidermis (Nguyen 2003). The various factors constituting this 'rhizosphere effect' vary with plant genotypes (Lupwayi and Kennedy 2007). Previous studies found that fungicide applications might affect root exfoliation, as fungicide can modify root physiology (Petit et al. 2008) and morphology (Baby et al. 2004; Ferreira et al. 2008). Pathogens, such as ascochyta blight, also influenced the physiology and morphology of plants, including chickpea (Pande et al. 2005). Different chickpea genotypes with different disease resistance have responded differently to ascochyta blight (Pande et al. 2005). Therefore, the occurrence of different bacterial communities under the same fungicide treatment in the rhizosphere of different chickpea cultivars is not surprising. It may reflect the different response of different plant genotypes to the chemicals they received or mediated via disease infection.

In this study, differences in VOCs between fungicide treatment and control chickpea in 2008 confirmed the modification effects of fungicide on chickpea physiology, which associated with antimicrobial activity of the plant or trigger responses in microorganisms

(Cruz et al. 2012). In this study, chickpea plants were impacted by ascochyta blight in 2008. Thus, it seems that plant response to the fungicide and resulting disease impact, rather than the fungicide application alone, is responsible for the differences found in the structure of the bacterial communities of fungicide treated and control chickpea rhizosphere.

I found treatment results varied between two experimental years, which maybe due to changes of environmental factors. Many environmental factors may affect growth of bacterial communities, such as differences in precipitation and temperature. The climatic conditions in 2008 and 2009 differed in total amount of precipitation during the growing season. Former studies reported that environmental changes modified the diversity and structure of either general bacterial (saprotrophic, pathogenic or mutualistic) community (Kennedy et al. 2005; Rasche et al. 2011) or specific symbiotic bacteria groups such as N_2-fixing bacteria (Welsh et al. 2009). In this study, these bacterial categories seem impacted by the activity of fungicide applications and/or fungal disease pressure (Stoddard et al. 2010).

3.6. Conclusion

Foliar fungicide application is commonly used in the control of fungal pathogens such as *Ascochyta rabiei* in chickpea crop. This study demonstrated that this practice impacted the composition of the general bacterial community in the rhizosphere soil of a chickpea crop, and that impact was be modified by chickpea genotype. Environmental factors, such as precipitation, may also influence the growth and composition of bacterial communities in chickpea rhizosphere. Therefore, selective use of genotypes could reduce the negative effects of chemicals used in the management of chickpea

production, which also need to combine with the consideration of environmental influence.

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4. Perface

The following chapter was accepted by the Journal of Applied Microbiology (C. Yang, C. Hamel, Y. Gan, V. Vujanovic. 2012. Non-target effects of foliar fungicide application on the rhizosphere: diversity of nifH gene and nodulation in chickpea field. Journal of Applied Microbiology. In Press. doi:10.1111/j.1365-2672.2012.05262.x). The work reported here is a demonstration of the fungicide application effects on N_2-fixing bacteria group in chickpea field. By using molecular technology denaturing gradient gel electrophoresis (DGGE) and cloning, this study reviewed the indirectly effects of fungicide application on the *nifH* gene composition in chickpea rhizosphere, through influencing host plant physiology.

C. Hamel and V. Vujanovic co-supervised this work. All co-authors reviewed the manuscript. K. Hanson helped to improve English. I planned this experiment, processed and analysed samples, and submitted clone samples for determination at Plant Biotechnology Institute (PBI), Saskatoon, Canada. I interpreted the data and prepared the manuscript for publication.

4. NON-TARGET EFFECTS OF FUNGICIDE ON THE RHIZOSPHERE: NITROGEN FIXING BACTERIAL COMMUNITY AND NODULATION IN CHICKPEA FIELD

4.1 Abstract

This study explores non-target effects of fungicide application on field-grown chickpea. Molecular methods were used to test the effects of foliar application of fungicide on the diversity and distribution of *nifH* genes associated with two chickpea cultivars, and their nodulation. Treatments were replicated four times in a split plot design in the field, in 2008 and 2009. Chemical disease control did not change the richness of the *nifH* genes associated with chickpea, but selected different dominant *nifH* gene sequences in 2008, as revealed by correspondence analysis. Disease control strategies had no significant effect on disease severity or *nifH* gene distribution in 2009. Dry weather conditions rather than disease restricted plant growth that year, suggesting that reduced infection rather than the fungicide is the factor modifying the distribution of *nifH* gene in chickpea rhizosphere. Reduced nodule size and enhanced N_2-fixation in protected plants indicate that disease control affects plant physiology, which may in turn influence rhizosphere bacteria. The genotypes of chickpea also affected the diversity of the *nifH* gene in the rhizosphere, illustrating the importance of plant selective effects on bacterial communities. I conclude that the chemical disease control affects nodulation and the diversity of *nifH* gene in chickpea rhizosphere, by modifying host plant

physiology. A direct effect of fungicide on the bacteria cannot be ruled out, however, as residual amounts of fungicide were found to accumulate in the rhizosphere soil of protected plants.

4.2 Introduction

Nitrogen limits plant growth in many ecosystems (Fiore et al. 2010). Biological nitrogen fixation (BNF) makes an important contribution to soil nitrogen (Zielke et al. 2005; Zhao et al. 2010) and improves plant productivity. Much research was devoted to understand the mechanisms of BNF in diazotrophs (Kessler and Leigh 1999; Petrova et al. 2000; Bashan and de-Bashan 2010; Oliveira et al. 2010) because of the importance of their contribution to the biosphere. Diazotrophs possess the enzymes nitrogenase and nitrogenase reductase carrying out N_2-fixation, i.e., the reduction of N_2 into NH_3. These N_2-fixing bacteria are diverse taxonomically and metabolically, but can be classified into three functional groups (Bürgmann et al. 2004). The free-living N_2-fixing bacteria contribute a relatively small proportion of the N input in ecosystems, due to the high energy requirement of the process. The associative N_2-fixing bacteria typically live on plant roots surface and can be quite active when fuelled by rhizodepositions. Symbiotic N_2-fixing bacteria trigger the formation of specialized organs such as root nodules within plant tissues and can fix considerable amounts of N_2. Symbiotic N_2-fixing bacteria are largely associated with leguminous plants (Lindström et al. 2010) and N_2-fixing leguminous crops are widely used to input BNF in agro-ecosystems throughout the world.

Denitrification of nitrogenous fertilizer residues into N_2O was identified as the main source of greenhouse gas emissions from farming activities (Janzen et al. 2006; Dyer et al. 2010; van Groenigen et al. 2010). Improved cropping systems involving N_2-fixing

crops in rotations can reduce the amount of greenhouse gas emissions and the environmental impact of agriculture (Gan et al. 2011). Therefore, N_2-fixing bacteria and BNF in cultivated fields are triggering much research interest.

Chickpea (*Cicer arietinum* L.) is the third most important leguminous crops worldwide. It is grown in the Mediterranean countries, Middle East, West Asia, Mexico and elsewhere (Kyei-Boahen et al. 2002; Pande et al. 2005; Millan et al. 2006). Chickpea is widely grown in rotation with wheat in southwest Saskatchewan and southeast Alberta, the driest part of the Canadian Prairie, where low precipitation, high diurnal temperature fluctuation, and sufficient heat lead to high quality grain. Chickpea could be an important source of nitrogen in wheat-based cropping systems of semi-arid regions of the world, but nodulation in this crop is sometimes reduced (Broughton and Perret 1999). Relatively few studies have examined the diversity of N_2-fixing bacteria in field-grown chickpea (Laranjo et al. 2008). *Mesorhizobium ciceri* and *M. mediterraneum* are known to nodulate chickpea (Nour et al. 1994; Nour et al. 1995). A later report showed a few more species able to nodulate chickpea (Laranjo *et al.* 2004). However, these results remain controversial (Laranjo et al. 2004; Rivas et al. 2007).

The poor reliability of nodulation in chickpea may be related to cropping practices rather than to plant genetics. Fungicides are used abundantly in chickpea crops to control Ascochyta blight, a devastating disease of this crop (Gan et al. 2006). Pesticide use may adversely affect agriculturally important microorganisms, including N_2-fixing bacteria, and reduce the performance of agroecosystems (Gaind et al. 2007). A close look at the effect of fungicide application on N_2-fixing bacteria in chickpea fields could help explain the variation in BNF activity observed in this crop and lead to the design of more sustainable cropping systems.

Molecular techniques have been used in research on N_2-fixing bacteria to resolve many important problems associated with traditional cultural methods (Hugenholtz et al. 1998). Among molecular tools, PCR-based profiling methods such as restriction fragment length polymorphism (RFLP) (Bürgmann et al. 2004) and denaturing gradient gel electrophoresis (DGGE) (Bürgmann et al. 2005) have been used to analyze the diversity of N_2-fixing bacterial communities. Nitrogenase reductase structural gene *nifH* (Howard and Rees 1996) was successfully used as a marker gene for BNF (Bürgmann et al. 2004) which yield good results. Therefore, I adopted a PCR-DGGE protocol using *nifH* as a target in order to: (1) improve knowledge on the N_2-fixing bacterial diversity in field-grown chickpea rhizosphere, and (2) define the effect of foliar disease control on chickpea rhizobacterial community.

4.3. Materials and methods

4.3.1 Experimental design and treatment application

A two-factor field experiment with split-plot design and four replicates was conducted in 2008 and 2009 at different locations of the Semiarid Prairie Agricultural Research Centre, near Swift Current, SK, Canada (latitude 50° 18’ N; longitude 107° 41’ W). The soil contained 3.6 kg ha^{-1} mineral N, 21.8 kg ha^{-1} sodium bicarbonate extractable P and 283 kg ha^{-1} available K in 2008, and 3.1 kg ha^{-1} mineral N, 12.6 kg ha^{-1} sodium bicarbonate extractable P and 210 kg ha^{-1} available K in 2009. The climatic conditions were drier in 2009 than 2008. Average precipitation during the growing season i.e. from April to September, was 59.3 mm $month^{-1}$ in 2008 and 35.6 mm $month^{-1}$ in 2009. Treatments consisted in a non-treated control and four different fungal disease control strategies (Table 3.1), involving Bravo® (Syngenta Crop Protection Canada Inc., Guelph, ON, a.i. chlorothalonil) and Headline® Duo (BASF Canada Inc., Mississauga, ON, a.i.

pyraclostrobin and boscalid), two fungicides commonly used to control Ascochyta blight in chickpea fields. These treatments were applied to two chickpea cultivars, CDC Luna and CDC Vanguard, representing two main types of chickpea, Kabuli and Desi, which differ in seed size, shape, color and nutrients content (Iqbal et al. 2006; Maheri-Sis et al. 2008). Nitragin Soil Implant + GC Peat-based Granular Inoculant, which contains a minimum of 100 million (1×10^8) viable cells of *Mesorhizobium ciceri* per gram of product, was applied at 5.6 kg ha^{-1}. The *nifH* gene sequences in this commercial inoculant were verified through DNA extraction, cloning and sequencing, using the procedure described below. The inoculant contained two *nifH* gene sequences. One was 97% similar to a *M. ciceri* (GenBank # EU267715.1) and another was 97% similar to *Bradyrhizobium* sp. (GenBank # CP000494.1).

4.3.2 Soil sampling

Rhizosphere soil samples were taken at chickpea harvest time in September of 2008 and 2009. Two soil cores (0-7.5 cm depth), were taken directly on the crop row using a 5-cm diameter manual soil sampler after sweeping away plant debris, and pooled to yield one composite sample per plot. Samples were brought to the laboratory, sieved through 2 mm, and placed in sealed plastic bags at -20 °C until molecular analysis.

4.3.3 Nodule sampling

Nodules were sampled when BNF usually peaks, i.e., one week after chickpea early-flowering stage. Since plots assigned to treatment II and treatment III were still treated exactly the same at that time, nodulation was not assessed in plots receiving treatment III. Five plants from each plot were removed using a shovel to minimize root disturbance and brought to the laboratory. Their roots were carefully cleaned with tap water to remove adhering soil and dried with paper towels before randomly collecting five

nodules from each plant (Rice and Clayton 1996). A nodulation score test based on internal color and size of nodules was then applied (Rice and Clayton 1996), using the pools of 25 nodules randomly collected from each plot.

4.3.4 Measurement of fixed nitrogen

The ^{15}N dilution technique was used to measure the amount of nitrogen fixed by chickpea under different treatments, using barley (*Hordeum vulgare* L.) as the non-fixing control plant (Jensen 1986). For this, a barley plot was planted beside each chickpea plot. $^{15}NH_4{}^{15}NO_3$ (10 atom%, ICON ISOTOPES, www.iconisotopes.com) was applied to both chickpea and barley microplots after plant emergence. Whole plants were collected at harvest time, taken back to the laboratory, cleaned with tap water to remove the soil attached on their surface, dried at 45 °C until constant weights, and finely ground. Plant nitrogen concentration and ^{15}N-to-^{14}N ratio were measured by mass spectrometry (V.G. Isotech, Aston Way, Middlewich, Cheshire, CW10 OHT, United Kingdom). The percentage and amount of nitrogen derived from air were calculated as:

$$N_2\ fixed = \frac{(atom\%^{15}\ Nexcess(non-\ fixing) - atom\%^{15}\ Nexcess(\ fixing)) \times total\ N(\ fixing\ crop)}{atom\%^{15}\ Nexcess(non-\ fixing)} \quad (4.1)$$

(Fried and Middelboe 1977).

4.3.5 Molecular analysis of diversity of *nifH* gene in chickpea rhizosphere

Raw DNA was extracted from chickpea rhizosphere soil using UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturers' instruction, and diluted 20 times before PCR amplification of a fragment (~ 450 bp) of the gene *nifH* using primers PloR / PloF (Poly et al. 2001). The PCR products were used as templates in a subsequent PCR using the same protocol except for

the primers, which were PloR and PloF-GC, i.e. PloF with a GC clamp at the 5' end. This amplification produced fragments of approximately 500 bp, which were used to construct a clone library and DGGE markers. UltraPure^{TM} DNase/RNase-Free Distilled Water (Invitrogen, Cat#10977015) was included in PCR instead of DNA template as negative control to exclude any risk of false DNA amplification.

A clone library of all the *nifH* gene sequences obtained from soil samples was created by pooling the PCR products amplified with primers PloR / PloF from soil samples (Renker et al. 2006). The DNA fragments were cloned into *Escherichia coli* (strain TOP 10) using the TOPO TA Cloning Kit (Invitrogen, Cat#K4575-J10) following the manufacturer's instructions. The transformed cells were plated onto solid Luria-Bertani (LB) medium containing ampicillin (50 μg ml^{-1}), incubated overnight at 37 °C, then transferred into a 96-well plate filled with liquid LB medium and sent for sequencing at the Plant Biotechnology Institute of the National Research Council of Canada, in Saskatoon, SK. The N_2-fixing bacteria associated with the experimental chickpea plants were identified based on the similarity of their *nifH* gene sequence to sequences deposited in GenBank, using the online program BLAST. Positive clones were subjected to PCR amplification using primer pair PloR / PloF-GC as mentioned above, and 10 μl of PCR product of each clone was submitted to DGGE (denaturing gradient gel electrophoresis), as described below, to locate a distinct migration position for each clone on the gel. Then, 10 μl of PCR product of each clone were pooled. This DGGE marker mix was loaded (40 μl) into a lane on each gel for the identification of the bands produced from experimental samples. All DNA were stored at -20 °C prior to analysis.

A DGGE protocol (Ma et al. 2005) was used to separate 20 μl of PCR products from each plot. Gels contained 6% (w/v) polyacrylamide (37:1 acrylamide / bis-acrylamide).

The linear gradient used varied from 35% to 65% denaturant, where 100% denaturing acrylamide was defined as containing 7 M urea and 40% (v/v) formamide. A 4-ml stacking gel containing no denaturants was added before polymerization was complete (~ 2 h). All DGGE separations were performed in a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at a constant temperature of 60 °C. After 10 min at 75 V, the voltage was lowered to 60 V for an additional 16 h. Gels were stained in 1 × Tris / acetic acid / EDTA buffer (TAE) containing 4 µl SYBR Safe DNA gel stain (Invitrogen) per 10 ml and visualized by UV illumination. Gel images were digitally captured by an OLYMPUS digital camera (SP-500UZ) in Multimage Light Cabinet (Alpha Innotech Corporation, San Leandro, USA) using a Sybr Safe filter.

4.3.6 Statistical analysis

Linear regression analysis was used to verify the relationship between chickpea yield, fixed nitrogen, and disease severity using SYSTAT 12. The *nifH* sequences detected in this study were aligned by BioEdit sequence alignment editor software (version 7.0.9.0.) using Clustal W multiple alignment algorithm. The diversity of *nifH* gene associated with the chickpea crops submitted to the different experimental treatments, as revealed by sequence profiling, was analyzed by MultiResponse Permutation Procedure (MRPP) using PC-ORD, and correspondence analysis using SYSTAT 12. Difference of nodulation scores were detected by ANOVA using SYSTAT 12. The Shapiro-Wilk test was used to verify the normality of distribution and homogeneity of variance prior to ANOVA. The Wilks' Lambda test, was used to detect significant treatment effects at 5% level in ANOVA using SYSTAT 12.

Table 4.1 Identity of the N_2-fixing bacteria living in chickpea rhizosphere, according to BLAST results.

Sequence designation#	Year	GenBank accession no. for closest match	Closest match from GenBank by BLAST[a]
1	2008 & 2009	AY583643.1	Uncultured bacterium clone SJ14 dinitrogenase reductase (*nifH*) gene, partial cds (98%)
2	2008 & 2009	AY819584.1	Uncultured bacterium clone M1b-77 dinitrogenase reductase (*nifH*) gene, partial cds (97%)
3	2008 & 2009	AB188121.1	*Azohydromonas australica nifH* gene for iron protein of nitrogenase, partial cds, strain:IAM 12664 (97%)
4	2008 & 2009	CP000494.1[b]	*Bradyrhizobium* sp. BTAi1, complete genome (97%)
5	2008 & 2009	AY196375.1	Uncultured nitrogen-fixing bacterium clone b1-HA3-7 nitrogenase iron protein (*nifH*) gene, partial cds (100%)
6	2008 & 2009	DQ995922.1	Uncultured nitrogen-fixing bacterium clone 57 dinitrogenase reductase (*nifH*) gene, partial cds (98%)
7	2008 & 2009	AB217474.1	*Sphingomonas azotifigens nifH* gene for dinitrogenase reductase subunit, partial cds (99%)
8	2008 & 2009	AY360976.1	Uncultured bacterium cluster *O NifH* (*nifH*) gene, partial cds (97%)
9	2008 & 2009	GU201868.1	*Rhizobium leguminosarum* strain Qtx-10-1 *NifH*-like (*nifH*) gene, partial sequence (97%)
10	2008 & 2009	AB542349.1	*Azospirillum* sp. TSA20c *nifH* gene for nitrogenase reductase, partial cds, strain: TSA20c (97%)
11	2008 & 2009	AM110711.1	*Azorhizobium caulinodans* partial *nifH* gene for putative nitrogenase, isolate T1 2 (98%)
12	2008 & 2009	EU267715.1[b]	*Mesorhizobium ciceri* strain USDA 3378 nitrogenase iron protein (*nifH*) gene, partial cds (97%)
13	2008 & 2009	DQ995918.1	Uncultured nitrogen-fixing bacterium clone 50 dinitrogenase reductase (*nifH*) gene, partial cds (98%)
14	2008 & 2009	GQ167280.1	*Mesorhizobium mediterraneum* strain USDA 3392 *NifH* (*nifH*) gene, partial cds; (99%)
15	2008 & 2009	AY583648.1	Uncultured bacterium clone SJ19 dinitrogenase reductase (*nifH*) gene, partial cds (97%)
16	2008 & 2009	AY630757.1	Uncultured bacterium clone SJY-2 dinitrogenase reductase gene, partial cds (100%)
17	2008 & 2009	GU083832.1	*Rhizobium giardinii* strain ZW7-1 nitrogenase reductase (*nifH*) gene, partial cds (99%)
18	2008 & 2009	EU770974.1	*Mesorhizobium septentrionale* CCBAU:03133 nitrogenase iron protein (*nifH*) gene, partial cds (100%)
19	2009	DQ995931.1	Uncultured nitrogen-fixing bacterium clone 67 dinitrogenase reductase (*nifH*) gene, partial cds (97%)
20	2009	AY907474.1	*Rhizobium gallicum* bv. *gallicum* strain IE988 nitrogenase reductase (*nifH*) gene, partial cds (98%)
21	2009	AY601060.1	Uncultured bacterium clone Langqian-3 dinitrogenase reductase (*nifH*) gene, partial cds (97%)
22	2009	DQ995922.1	Uncultured N_2-fixing bacterium clone 57 dinitrogenase reductase (*nifH*) gene, partial cds (98%)
23	2009	GQ503352.1	*Mesorhizobium ciceri* strain Rcd301 dinitrogenase reductase (*nifH*) gene, partial sequence (100%)

Note: [a] Sequence similarity values below 97% are not considered to be identical.
[b] Sequeces belonging to the strains of the commercial inoculant used.

4.4 Results

4.4.1 Diversity of nifH gene fragments as affected by treatments

A total of 23 different *nifH* gene sequences were detected by the PCR-DGGE analysis method (Table 4.1), and *nifH* sequences closely affiliated to *M. cicer* were found. However, eight sequences were related to other symbiotic and non-symbiotic genera and eleven sequences showed close similarity to uncultured species, revealing a high diversity of *nifH* gene in chickpea rhizosphere soil. In 2008, significant effects of cultivar ($P < 0.001$) on community structure (Fig. 4.1) were detected by MRPP analysis, revealing a selective effect of chickpea genotype on the diversity of rhizosphere *nifH* gene. No significant effects of genotypes on *nifH* gene diversity were found in 2009 (Fig. 4.1).

4.4.2 Distribution of nifH gene in chickpea rhizosphere as affected by treatments

Results of Correspondence Analysis indicate that both disease control treatments and cultivars influenced the distribution of dominant *nifH* genes in 2008 (Fig. 4.1). A significant relationship was found between *nifH* gene sequences and the combinations of disease control and cultivar treatments in 2008 ($P = 0.014$). The sequence related to Clone b1-HA3-7 (sequence designation# 5 as shown in Table 4.1) was associated with CDC Luna treatment III and the sequence related to *Azospirillum* sp. (sequence designation# 10), with CDC Luna treatment II. The *nifH* gene sequence closely affiliated to *A. caulinodans* (sequence designation# 11) was frequent in the rhizosphere of CDC Vanguard control, but rare in the rhizosphere of CDC Luna (Fig. 4.1). In 2009 (Fig. 4.2), the relationship between *nifH* gene distribution and treatments was non-significant.

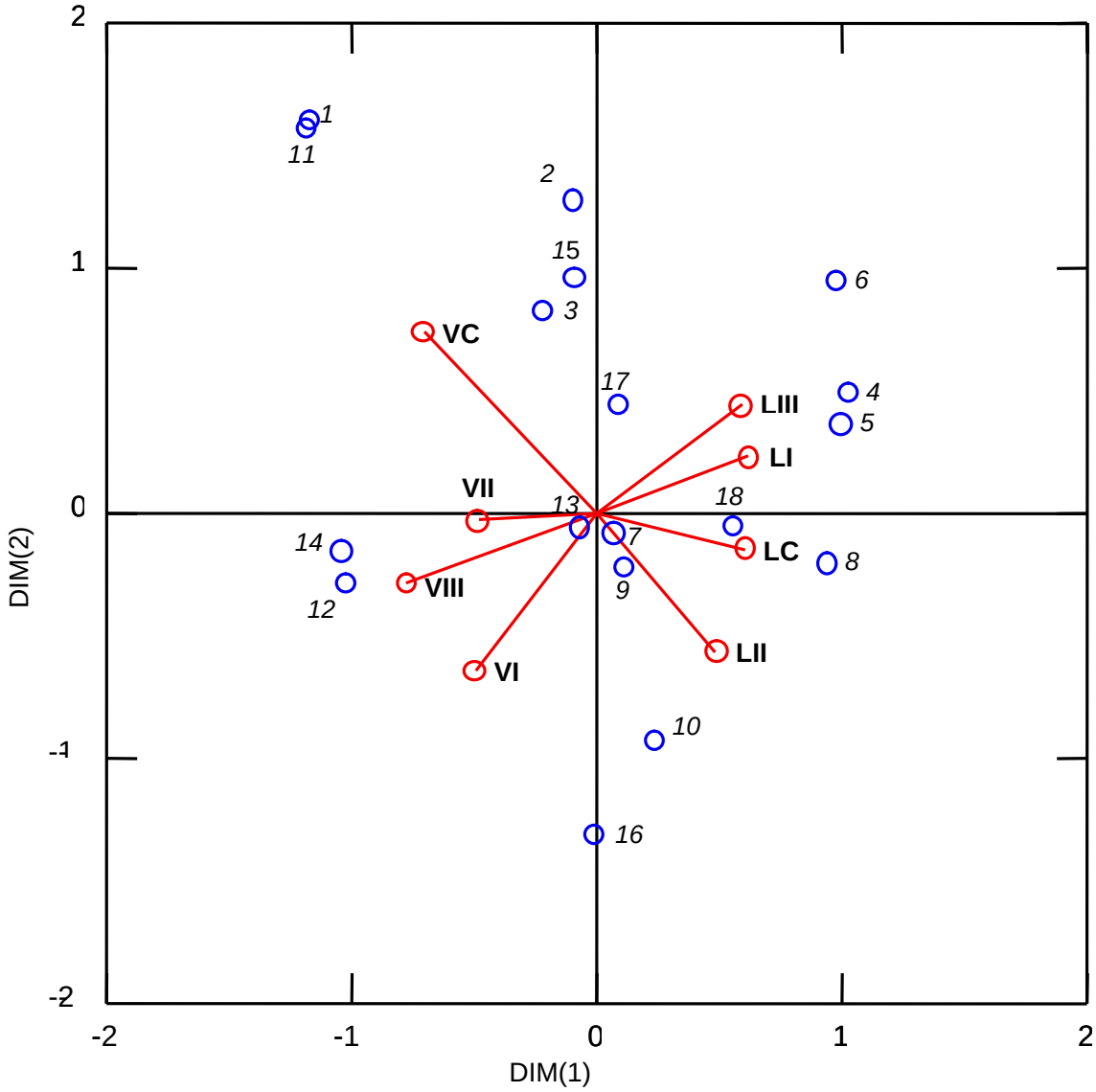

Figure 4.1 Correspondence analysis of relationships between disease control treatments and identified dominant N_2-fixing bacteria in the rhizosphere of both chickpea cultivars in 2008, as revealed by *nifH* gene. C: control; I, II and III: increasing intensity of fungicide application; V: CDC Vanguard; L: CDC Luna; Numbers correspond to the identified N_2-fixing bacteria shown in Table 4.1. $P = 0.014$, N = 32.

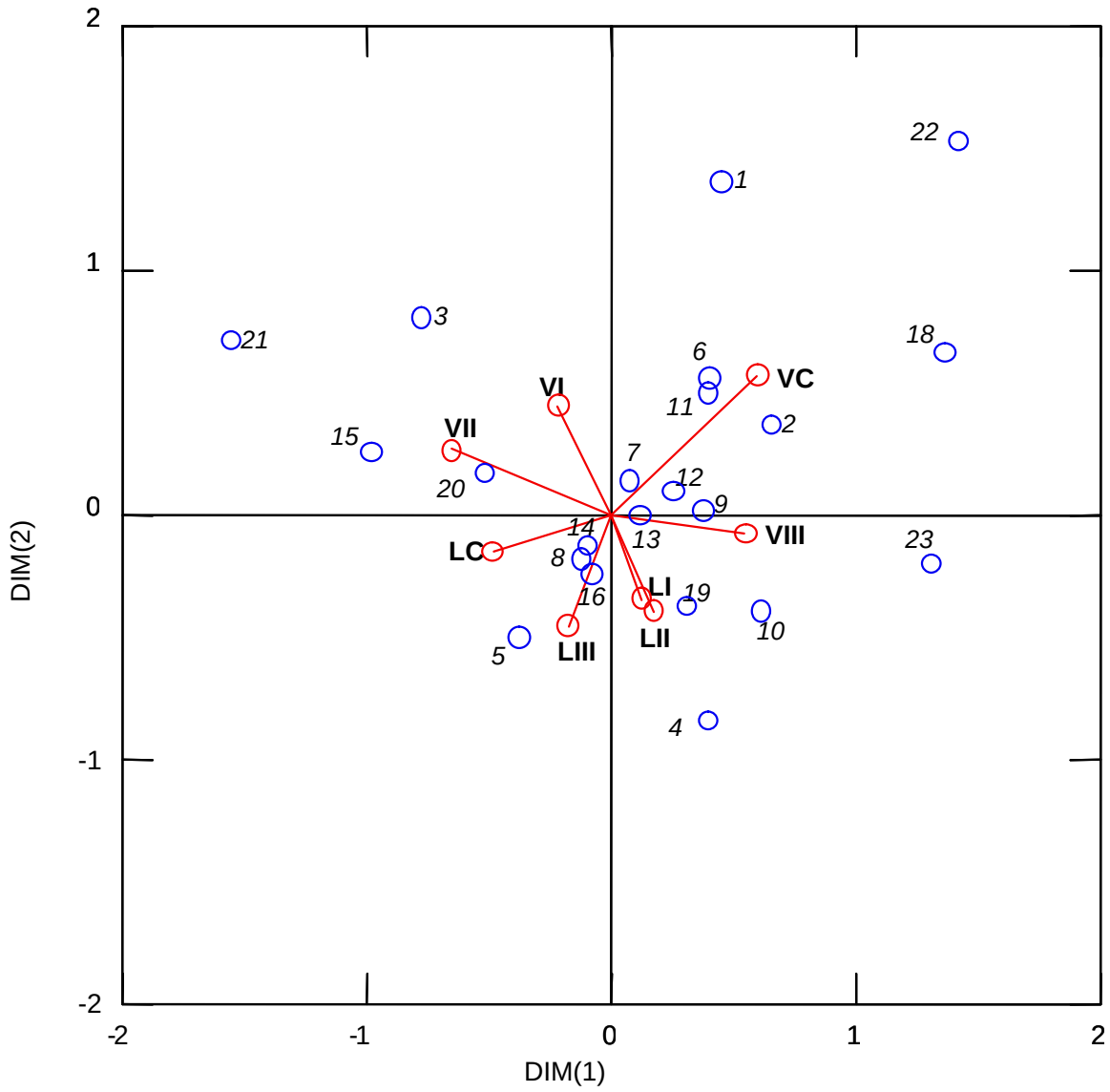

Figure 4.2 Correspondence analysis of relationships between disease control treatments and identified dominant N_2-fixing bacteria in the rhizosphere of both chickpea cultivars in 2009, as revealed by *nifH* gene. C: control; I, II and III: increasing intensity of fungicide application; V: CDC Vanguard; L: CDC Luna; Numbers correspond to the identified N_2-fixing bacteria shown in Table 4.1. $P > 0.05$, N = 32.

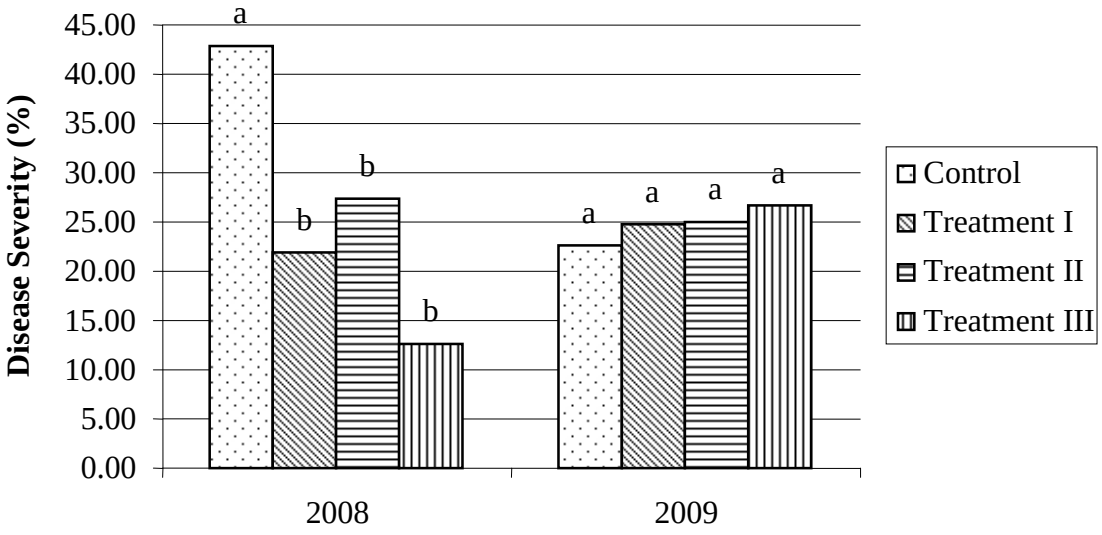

Figure 4.3 Effects of disease control application on disease severity in chickpea field in 2008 and 2009. (P = 0.003 and 0.719 in 2008 and 2009, respectively). Different low case letters indicates significantly different means, according to Wilks' Lambda test (α = 0.05, n = 16).

Table 4.2 Effects of cultivar, disease control strategy, year and their interacting effects on nodulation scores, fixed N and grain yield in chickpea field, according to ANOVA

Factors		Nodulation Scores		Fixed N (kg ha^{-1})		Yield (kg ha^{-1})	
		Mean±SE	P value	Mean±SE	P value	Mean±SE	P value
Cultivar (C)	Luna	6.4±0.3	ns	12.5±1.5	<0.001	1357±93	<0.001
	Vanguard	6.9±0.2		18.6±1.5		1908±90	
Disease control (D)	Control	7.6±0.3	<0.001	12.4±1.9	0.04	1339±124	<0.001
	I	6.5±0.3		17.2±2.1		1630±124	
	II	5.8±0.4		17.1±2.3		1668±133	
	III	/		15.6±2.6		1892±176	
Year (Y)	2008	7.0±0.2	0.047	21.4±1.4	<0.001	2030±91	<0.001
	2009	6.3±0.3		9.7±0.9		1235±57	
C×D		/	ns	/	ns	/	ns
C×Y		/	ns	/	ns	/	ns
D×Y		/	ns	/	ns	/	<0.001
C×D×Y		/	ns	/	ns	/	0.02

Note: ns means non-significant at $\alpha = 0.05$; $N = 64$.

Table 4.3 Relationship among grain yield, fixed N and disease rating in chickpea field in 2008 and 2009.

	2008			2009		
	Yield	Fixed N	Disease	Yield	Fixed N	Disease
Yield	1.000			1.000		
Fixed N	0.515**	1.000		0.897**	1.000	
Disease	-0.761**	-0.263ns	1.000	-0.161ns	-0.145ns	1.000

Note: numbers in the table are Person correlation coefficients, ** Means $P < 0.001$, ns means not significantly different at $P < 0.05$ according to Linear Regression Analysis; $N = 32$.

The *nifH* gene related to *M. ciceri* contained in the commercial inoculant applied was frequently detected in the rhizosphere of protected CDC Vanguard in 2008, but rarely detected in CDC Luna rhizosphere (Fig. 4.1). The *nifH* gene with high similarity to *Bradyrhizobium* sp. contained in the inoculant was frequent in the rhizosphere of CDC Luna, but rare in that of CDC Vanguard, both in 2008 and 2009.

4.4.3 Fungicide effects on biological N_2 fixation

ANOVA results showed that chickpea nodulation scores were significantly decreased with an increase in fungicide application intensity (Table 4.2), indicating that disease control treatments reduce chickpea nodulation. The concurrent enhancing effect of disease control on BNF (Table 4.2) suggested that disease control negatively impacted nodule size but not their functions. Nodule scores and N_2-fixation were higher in 2008 than in the drier 2009 (Table 4.2).

4.4.4 Fungicide effects on disease control and yield of chickpea

A significant negative correlation between yield and disease severity in 2008 (Table 4.3) revealed the importance of disease outbreak as a yield limiting factor that year. Strong disease control x year interactions influenced yield (Table 4.2) reflecting that Ascochyta blight impacted plant productivity only in 2008 (Fig. 4.3), when wetter weather was conducive to early disease outbreak. In 2009, low disease pressure made disease control useless and no effect of chemical disease control strategies on disease severity was detected (Fig. 4.3). By contrast, no disease control x year interaction was found to influence nodulation score (Table 4.2) suggesting that fungicide application per se, rather than disease control, is the cause of reduced nodulation scores in fungicide treated plants.

4.5 Discussion

This study revealed an important diversity of *nifH* gene related to free living diazotrophs in chickpea rhizosphere. Most of *nifH* gene sequences affiliated to N_2-fixing bacterial species detected in the chickpea rhizosphere were uncultured and non-symbiotic, indicating that free-living N_2-fixing bacteria may also be involved in N cycling in Canadian Prairie agroecosystems. *Azohydromonas australica* was reported earlier as free living N_2-fixing bacteria in sorghum field (Xie and Yokota 2005) and isolated later as endophytic bacteria from storage root of sweet potato (Terakado-Tonooka et al. 2008), and *Sphingomonas azotifigens* was reported as free living N_2-fixing bacteria in rice fields (Xie and Yokota 2006). The presence and contribution of these free-living N_2-fixing bacteria to BNF in chickpea field is not documented. The free-living bacteria *Azospirillum* sp., however, was recently reported in chickpea field, where they fixed N_2 and promoted plant growth when co-inoculated with *Azotobacter* spp. and *Pseudomonas* spp. (Rokhzadi and Toashih 2011).

The nifH gene diversity in the fields studied may be larger than reported here. Sequence analysis of the bands excided from the DGGE gel gave an insight into the dominant microbial taxa. Results presented should represent only the tip of the "iceberg" of *nifH* gene diversity in chickpea rhizosphere, which would be revealed by more sensitive methods of massively parallel sequencing.

The results suggests a contribution of the free-living N_2-fixing bacterial community to the growth of chickpea mediated through BNF, but also through the promotion of other plant growth promoting bacteria. Plants strongly compete with microorganisms for nitrogen (Hodge et al. 2000) and an actively growing crop plant may importantly reduce soil N availability to microorganisms. However, free-living N_2-fixing bacteria may

reduce N starvation in the rhizosphere microbial community of actively growing crop plants.

The effects of disease control on BNF can be direct or indirect. Headline® Duo is a systemic fungicide i.e., it is absorbed by leaves and systemically moves within the plant. In this study, rhizosphere soil samples contained sizeable residual amounts of boscalid, an active ingredient of Headline® Duo. Disruption of the electron respiration chain in microbial cells by boscalid (Wang et al. 2009b) and pyraclostrobin (Bartlett et al. 2002), the other active ingredient of Headline ® Duo, has been reported.

Whereas, fungicide application impacts the rhizosphere N_2-fixing community (Gaind et al. 2007), here, this community appeared to be only mildly influenced. Based on MRPP analysis, application of Bravo® and Headline® Duo had insignificant influence on the diversity of *nifH* gene. However, CA detected changes induced by disease control on the structure of the *nifH* gene diversity in 2008, when control plants were severely impacted by Ascochyta blight, but not in 2009, which was dry in early summer and when the disease appeared only late in the season. The production of bioactive volatile compounds by chickpea leaves and roots was much higher in diseased than in fungicide protected chickpea in 2008 (Cruz et al. 2012), supporting the involvement of plant defense mechanisms rather than a direct effect of fungicide on *nifH* gene diversity. Thus, it seems that disease control is responsible for the differences found in the structure of the N_2-fixing communities between protected and control rhizospheres in chickpea. Studies have shown that the composition of N_2-fixing bacterial communities is affected by both soil conditions (Fierer and Jackson 2006) and their associated plant (Normand et al. 2007; Wang et al. 2009a).

Nodulation, in contrast to the rhizosphere N_2-fixing bacterial community, seemed directly impacted by the fungicide application. Disease control reduced nodule size similarly in both years whether or not Ascochyta blight influenced the host plant. This concurs with former research showing fungicide-related modification in the rhizosphere in response to changes in plant photosynthesis (Petit et al. 2008), morphology (Baby et al. 2004) and root growth reduction (Ferreira et al. 2008). Fungicide treated plants fixed more N_2 than non-treated plants in this study, thus, the presence of small nodules, here, does not reflect reduced N_2-fixing activity in protected plants. It may indicate that plants had allowed more bacteria entry in the recent past, perhaps after an episode of nodule shedding upon fungicide application, or that chemical protection influenced the process of nodulation in a way that increased the number of points of entry of symbiotic bacteria. Increased or changed chemical composition of protected plants root secretions could explain the changes observed in the composition of the rhizosphere N_2-fixing bacterial communities of chickpea. Legumes produce specific chemical signals influencing symbiotic N_2-fixing bacteria (Geurts et al. 2005) and perhaps other bacteria.

Genotype effects on N_2-fixing bacterial community in chickpea rhizosphere were important, and confirm the results of a previous study on host range in rhizobium isolates (Ampomah et al. 2008). The selective effects of genotype on N_2-fixing bacteria could be due to differences in root secretion between cultivars, as proposed earlier (Lupwayi and Kennedy 2007). The growth and population densities of rhizosphere bacteria can be increased by large amounts of root secretion, sloughing-off of root cap cells, and senescing root epidermis in rhizosphere soil (Nguyen 2003). The symbiotic N_2-fixing bacteria could also be influenced by differences in the symbiotic signaling physiology of the two chickpea genotypes. Specific flavonoids produced by legumes

attract specific rhizobia to their root hairs, and the rhizobia in turn, produce the "nod factors" that induce root hair infection and nodule formation (Geurts et al. 2005). Differences in the signaling system of different chickpea genotypes could result in differences in the nodulation pattern between the plants or in the structure of the N_2-fixing bacterial communities in their rhizosphere.

Foliar fungicide application to control Ascochyta blight in chickpea crop is a widespread agronomic practice. Overall, the results of DGGE and clone libraries revealed that disease control strategies can modify nodulation and the composition of the nitrogen fixation associated gene fragments in rhizosphere apparently through its effect on the crop plants. This effect of disease control strategies tested in this study was relatively small, and may have been modified by environmental conditions. Environmental influences have lower impact than fungicide application on the process of nodulation, which is more intimately related to the plant than rhizosphere composition and differently regulated. Environmental conditions, conducting to disease, trigger plant defence reactions seemingly impacting free-living N_2-fixing bacteria.

4.6 Conclusion

Foliar fungicide application to control ascochyta blight in chickpea field is a widespread agronomic practice that can modify nodulation and the composition of the nitrogen fixation associated gene fragments in rhizosphere apparently through its effect on the crop plants. This effect of fungicide application was relatively small in this study, and modified by environmental conditions. Environmental influences have lower impact than the effects of fungicide application on the process of nodulation, which is intimately related to the plant.

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5. Perface

The work is under the review of Microbial Ecology (Manuscript ID: MECO-2011-0468). This study compares the effects of different pulse crops on the rhizobacterial community structure in pulse-cereal rotation. The work offered an opportunity to look into the influence of these cropping practices on rhizobacterial community and their functioning. The work was co-supervised by C. Hamel and V. Vujanovic, while Y. T. Gan (co-author) provided his feedback for improving the manuscript. I prepared a research proposal, plan sampling, lead the sampling team, processed samples, analyzed data and prepared the manuscript.

5. PULSE-CEREAL ROTATION: EFFECTS OF DIFFERENT PULSE CROPS ON THE COMMUNITY STRUCTURE AND FUNCTIONALITY OF RHIZOBACTERIA

5.1 Abstract

Crop rotation is a crop production strategy which is widely adapted for the important benefits it brings to the cropping system. This study determined the differential influence of chickpea and yellow pea as previous crops on their rhizobacterial communities, and potential impact on the growth of durum wheat as a following crop. The effects of pulse crops on the composition of their associated rhizobacterial community were examined using 454 GS FLX amplicon pyrosequencing. When inoculated on greenhouse-grown durum wheat, the 2008 rhizobacterial communities selected by yellow pea and CDC Luna chickpea in 2008 promoted durum wheat growth better than that by CDC Vanguard or CDC Frontier. Based on Chao 1 and ACE richness indices and Shannon (*H'*) diversity indices, yellow pea reduced the diversity of the rhizobacterial community at the end of the growing season, in 2008. These biodiversity indices also revealed differences in the influence of the three chickpea genotypes on rhizobacteria at the same time. In 2009, however, the effect of crop on the rhizobacterial community was mitigated and inoculation with the communities selected by the field-grown pulse crops had no significant effects on durum wheat growth. Thus, I conclude that different previous crops can select different rhizobacterial communities, which might trigger differences in the growth of a crop following in rotation. The strength of this rotation effect is influenced by environmental factors such as precipitation.

5.2 Introduction

The inclusion of pulse crops in rotation with cereals provides multiple benefits to cropping systems (Kirkegaard et al. 2004; Pala et al. 2007; Ryan et al. 2008a). In particular, pulse-cereal rotation can improve water use efficiency (Pala et al. 2007), soil aggregation (Masri and Ryan 2006), soil N availability (Pierce and Rice 1988), soil organic matter level (Ryan et al. 2008b), can break disease cycles (Karlen et al. 1994), and increase crop yield (Ryan et al. 2010). Sometimes, however, rotation effects can only be explained by the involvement of biotic processes unrelated to plant diseases (Kirkegaard et al. 2008).

Crop plants have a pivotal influence on rhizobacteria and different crops may associate with functionally different rhizosphere communities (Lupwayi and Kennedy 2007). Plants provide different rhizosphere environments and select specific rhizobacteria (Lugtenberg and Kamilova 2009), in turn, these bacteria can affect plant growth differently in either positive or negative ways. Various mechanisms can be involved in the enhancement of plant growth by plant-growth-promoting-rhizobacteria (PGPR). The PGPR may synthesize phytohormones (Sant'Anna et al. 2011), fix nitrogen (Peoples and Craswell 1992), solubilize phosphorus (Nannipieri et al. 2008), increase plant tolerance to extreme environmental conditions (Holzinger et al. 2011), and promote plant health through antibiotic production (Van Loon 2007) or competition with pathogens for niches and nutrients (Lucy et al. 2004). By contrast, other rhizobacteria repress plant growth by acting as a carbon drain and causing diseases, galls and tumours on plants (Spaepen et al. 2009). The composition of the rhizobacterial community is an important determinant of plant success that may vary with plant genotype.

The seeds of chickpea (*Cicer arietinum* L.) are a major source of protein in human nutrition (Ibrikci et al. 2003). Growing chickpea also provides environmental benefits (Gan et al. 2011; Lindström et al. 2010). Chickpea is well adapted to arid and semiarid areas worldwide (Kyei-Boahen et al. 2002; Millan et al. 2006; Pande et al. 2005) where the crop is often grown before wheat (*Triticum aestivum* L.) in rotations. However, the benefits derived from a previous chickpea crop are often modest as compared with those associated with a previous pea (*Pisum sativum* L.) crop (Miller et al. 2003b). The relatively poor performance of chickpea as a rotation crop in pulse-wheat rotation system is unexplained. Chickpea has an indeterminate growth habit (Anbessa et al. 2007) and grows longer than other pulse crops, often well into fall (Gan et al. 2008). The influence of chickpea on rhizobacteria may differ from that of other pulses, and affect functional rhizobacterial groups (Roesti et al. 2006). Chickpea crops could degrade soil microbial quality with impact on the following crop.

Therefore, the objective of this study was to assess the difference in the composition of rhizobacterial communities associated with various pulse crops and to determine the influence of these rhizobacterial communities on wheat growth. I hypothesized that: 1) the rhizobacterial communities associated with chickpea and pea differ, and 2) the rhizobacterial communities selected by different pulse crops can influence differently the growth of wheat.

5.3 Materials and methods

5.3.1 Experimental design and location

A two-factor field experiment (crop × sampling time) was set up in a randomized complete block design at the South Farm of the Semiarid Prairie Agricultural Research Centre (SPARC), in the Canadian Great Plains (latitude 50° 18’ N; longitude 107° 41’

W) in 2008, and was repeated in 2009. The soil of the sampling sites were Orthic Brown Chernozem containing 3.60 kg N ha^{-1}, 21.81 kg P ha^{-1}, and 283 kg K ha^{-1} in 2008, and 3.06 kg N ha^{-1}, 12.58 kg P ha^{-1}, and 210 kg K ha^{-1} in 2009. Average monthly precipitation during the growing season (1 April to 30 September) were recorded by a meteorological station located about 300 m from the experimental sites. The rhizobacterial communities associated with three chickpea cultivars (CDC Frontier, CDC Luna and CDC Vanguard) and yellow pea (CDC Handel) was examined at two different sampling times (July and September). Each treatment was replicated four times.

5.3.2 Soil sampling

Rhizosphere soil was sampled twice per growing season: (1) in early July when pea plants had reached maturity, and (2) in late September when chickpea was matured. The first centimeter of the soil surface was removed to eliminate plant debris, five plants were dug from each plot using a shovel, after gently shaken off bulk soil, plant roots with adhering rhizosphere soil were pooled to yield one composite sample per plot. The samples were put on ice and taken to the laboratory where the adhering soil was carefully brushed off the roots, sieved through 2 mm and stored in plastic bags at -20°C. Samples collected in September were divided into two subsamples. Within two days of soil sample collection, one of the subsamples was used to examine the functionality of the pulse rhizobacterial community using a bioassay conducted in the greenhouse. Another set of rhizosphere soil subsamples were stored for further molecular analysis of the taxonomic diversity of rhizobacterial communities.

5.3.3 Greenhouse experiment

The greenhouse experiment was used to test the influence of rhizosphere microorganisms selected by the field-grown pulse crops grown in the field experiment in

2008 and 2009, using rhizosphere soil collected from both chickpea and yellow pea plots in September. Durum wheat (*Triticum turgidum* var *durum* Desf.), a typical crop planted after chickpea in rotation series on the Canadian Great Plains, was used for the test. The seeds of durum wheat cultivar AC Avonlea were surface sterilized with a mixture of 70% ethanol and 30% hydrogen peroxide (1:1) for 2 min and rinsed several times with sterile distilled water. Surface sterilized seeds were pre-germinated overnight at 28°C in the dark. Five seeds at the same germination stage were selected, each seed was covered with five grams of chickpea or pea rhizosphere soil collected from the experimental field plots in September and placed into 1-L pots filled by pasteurized field soil (Figure 5.1). A non-inoculated control received only pasteurized soil. Plants were thinned to one plant per pot after emergence. There were five treatments in total: inoculation with rhizosphere soil from chickpea cultivars CDC Frontier, CDC Luna, CDC Vanguard, or from yellow pea, and a non-inoculated control. Treatments were replicated four times. The pasteurized soil was taken from a cultivated Brown Chernozem located 30 km northwest of Swift Current, SK, Canada. This soil had a loamy sand texture, a pH of 6.5, and an EC of 0.48 dS m^{-1}, it contained 19.7 mg kg^{-1} NH_4-N, 14.1 mg kg^{-1} NO_3-N, 21.3 mg kg^{-1} P (sodium bicarbonate extractible) and 324.5 mg kg^{-1} K after pasteurization at 80°C for 3 h.

Pots were arranged in a complete randomized design in the greenhouse and grown under a 15°C / 22°C (night/day) temperature regime and a relative humidity level of 75%. Natural day light was supplemented with high intensity discharge lamps (Alto 400 watt low pressure sodium, Philips, Somerset, NJ, USA) providing photosynthetically active radiation for 15 h day^{-1}. Plants received an equal amount of water every two days, as needed. Pots were re-randomized weekly to give all pots equal chance to be exposed

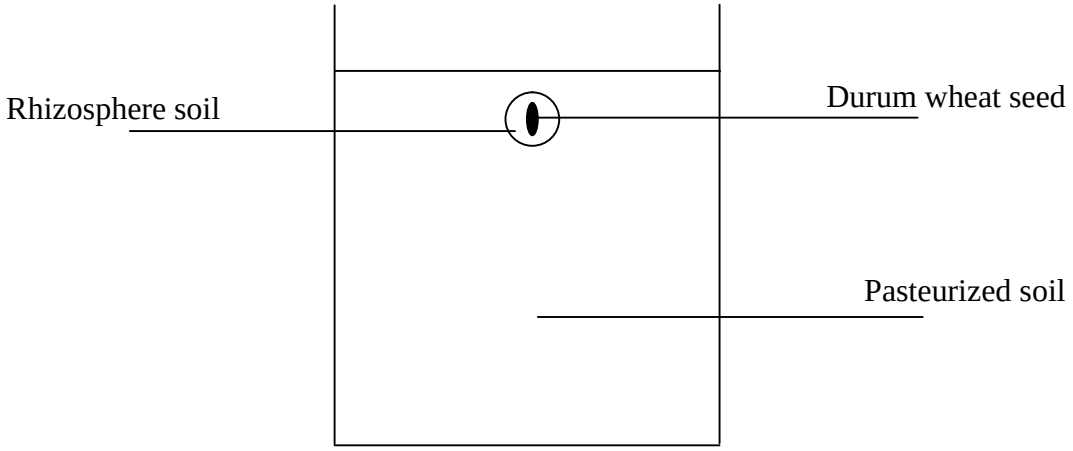

Figure 5.1 Experimental setup to assess the plant growth promoting ability of chickpea rhizosphere organisms on durum wheat in the greenhouse.

to particular micro-environmental conditions that may exist on the greenhouse bench. After one month growth, both fresh and dry weights (drying at 45°C for 2 days) of plant shoots and roots were recorded (OHAUS AV2101C Scale, NJ, USA).

5.3.4 Soil DNA extraction and PCR for tag-encoded 454 GS FLX bacterial amplicon pyrosequencing

Raw DNA was extracted from 0.4 g of rhizosphere soil using UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's protocol, and diluted 20 times after extraction. Diluted DNA was subjected to Polymerase Chain Reaction (PCR) using the16S rDNA-target primers 968f / 1401b amplifying an approximately 450 bp fragment of the bacterial universal gene. 454 Life Science's A or B adaptors were fused to the 5' end of the forward and reverse primers and unique multiplex identifier sequences (MID) were added between adaptor A and the forward primer to relate sequences to samples (Table 3.2). Platinum® PCR SuperMix (Cat. No. 11306-016, Invitrogen™) was used in PCR reactions conducted in an Veriti™ 96-well Fast Thermal Cycler (Applied Biosystems, California, USA) under the following conditions: 4 min initial denaturation at 94°C; 30 cycles of 45 s denaturation at 94°C, 45 s annealing at 56°C and 1 min elongation at 72 °C; plus a final 15 min elongation at 72 °C.

All PCR products were purified on agarose gel. Briefly, PCR products were run in 1% (w / v) agarose gel under 65 V for 1 h. Gel pieces containing visible bands of target size were then excised with a sterile blade. Each piece of gel was put into a sterile centrifuge tube with 30 µl TE buffer (1×dilution), vortexed for 1 min and placed at 4 °C over night for extraction. The concentration of purified PCR products in TE buffer was measured with Nano Drop-1000 (Thermo Scientific®, Wilmington, USA). The PCR products with

different multiplex identifiers were submitted for pyrosequencing at Génome Québec (Montréal, Québec, Canada) in pools of 16 samples.

5.3.5 Bioinformatics and statistical analysis

All sequence data were first edited to remove primer, MID and adaptor sequences using Mothur V.1.15.0 (Schloss et al. 2009). OTUs at 3% dissimilarity level were determined by comparing the sequences with the Silva database (http://www.arb-silva.de/) using Mothur (Wu et al. 2010). Chao 1 and ACE richness estimating indices, and Shannon (*H'*) diversity index were calculated based on the number of OTUs. Hierarchical cluster analysis was done using R (R Development Core Team 2009) with "gplots" (Warnes 2006) and "RColorBrewer" packages (Neuwirth 2007). The effects of pulse genotypes on Chao 1 and ACE richness estimating indices, and Shannon (*H'*) diversity index were tested by ANOVA in SYSTAT 12.0. The effect of inoculation with pulse rhizosphere soil on durum growth was also tested by ANOVA. The significance of differences between the effects of pea rhizosphere soil and chickpea rhizosphere soils was tested by Contrast analysis, using Network JMP (version 3.2.6), and the significance of differences between treatment means were further assessed using Fisher-LSD tests at $P < 0.05$.

5.4 Results

5.4.1 Growth promotion potential of pulse rhizosphere

Durum wheat inoculated with pulse rhizosphere soils collected in September 2008 showed significantly higher shoot and root fresh weights, as compared with the non-inoculated control (Figure 5.2). Durum wheat had higher shoot and root weight (P = 0.002 and 0.046, respectively) after inoculation with yellow pea than with chickpea rhizosphere soils, as revealed by contrast analysis. The growth-promoting effect of

rhizosphere soil not only varied between chickpea and yellow pea, but also among the different cultivars of chickpea. In particular, only CDC Luna and yellow pea rhizosphere soil significantly increased shoot dry weight as compared to the non-inoculated control. However, no such significant effects of inoculation with rhizosphere soils were found in 2009 (Figure 5.2).

5.4.2 Rhizospheric bacterial communities associated with field-grown pulse crops

The 454 pyrosequencing platform produced about 104,910 raw reads of bacterial 16S amplicons. Before the determination of OTUs, DNA fragments shorter than 400bp were removed, and identical sequences in raw reads were combined as one unique sequence, to avoid overestimation of OTUs. After trimming, 37,471 unique sequences were used for further determination of bacterial OTUs and phyla making up the communities. Among all identified bacterial phyla, Actinobacteria was dominant, comprising 16% - 39% of total identified OTUs (Figure 5.3). The Proteobacteria (19% - 24%) and Acidobacteria (15% - 23%) were the other two dominant phyla. The abundance of these phyla increased from July to September in 2008 (Figure 5.3), but decreased over the same period in 2009 (Figure 5.3), and this decrease in abundance was more conspicuous in the Proteobacteria ($P = 0.025$) and Actinobacteria ($P < 0.001$) than in the Acidobacteria ($P = 0.244$).

Contrasting results obtained in the two experimental years concurred with contrasting weather conditions in 2008 and 2009. Precipitation during the growing season was lower in 2009 than in 2008 (Figure 5.4). Precipitation from April to September in 2008 was 45% more than the 30-year average for the region (243 mm), but 25% less than the average in 2009.

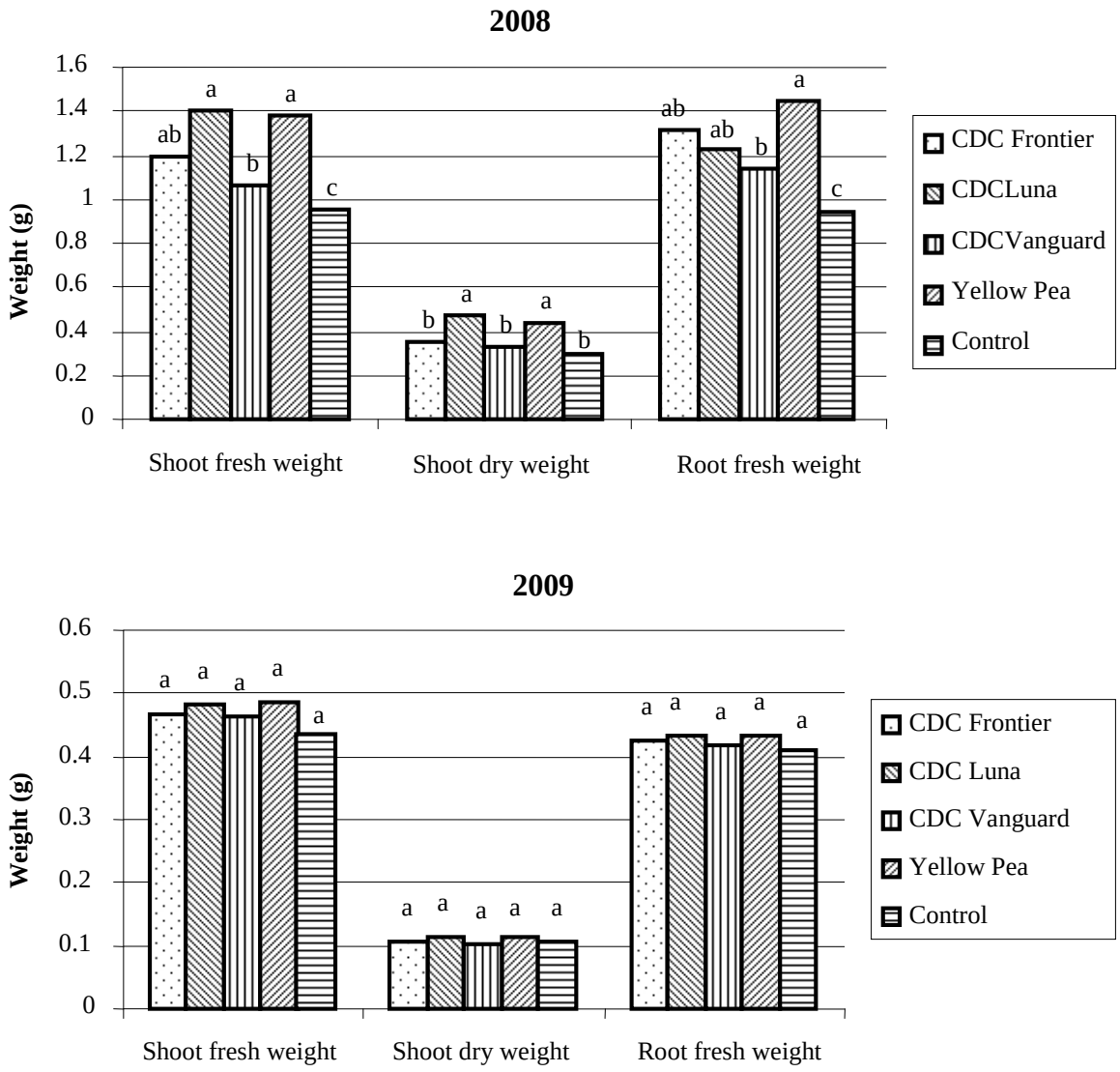

Figure 5.2 Shoot and root mass of wheat plants as influenced by inoculation with yellow pea and three chickpea rhizosphere soils in 2008 ($P < 0.0001$ for shoot and root mass) and 2009 ($P = 0.334$, 0.578 and 0.338 for shoot fresh weight, shoot dry weight and root fresh weight, respectively). Different small letters indicates significant differences based on Fisher-LSD test at $P < 0.05$ ($n = 4$).

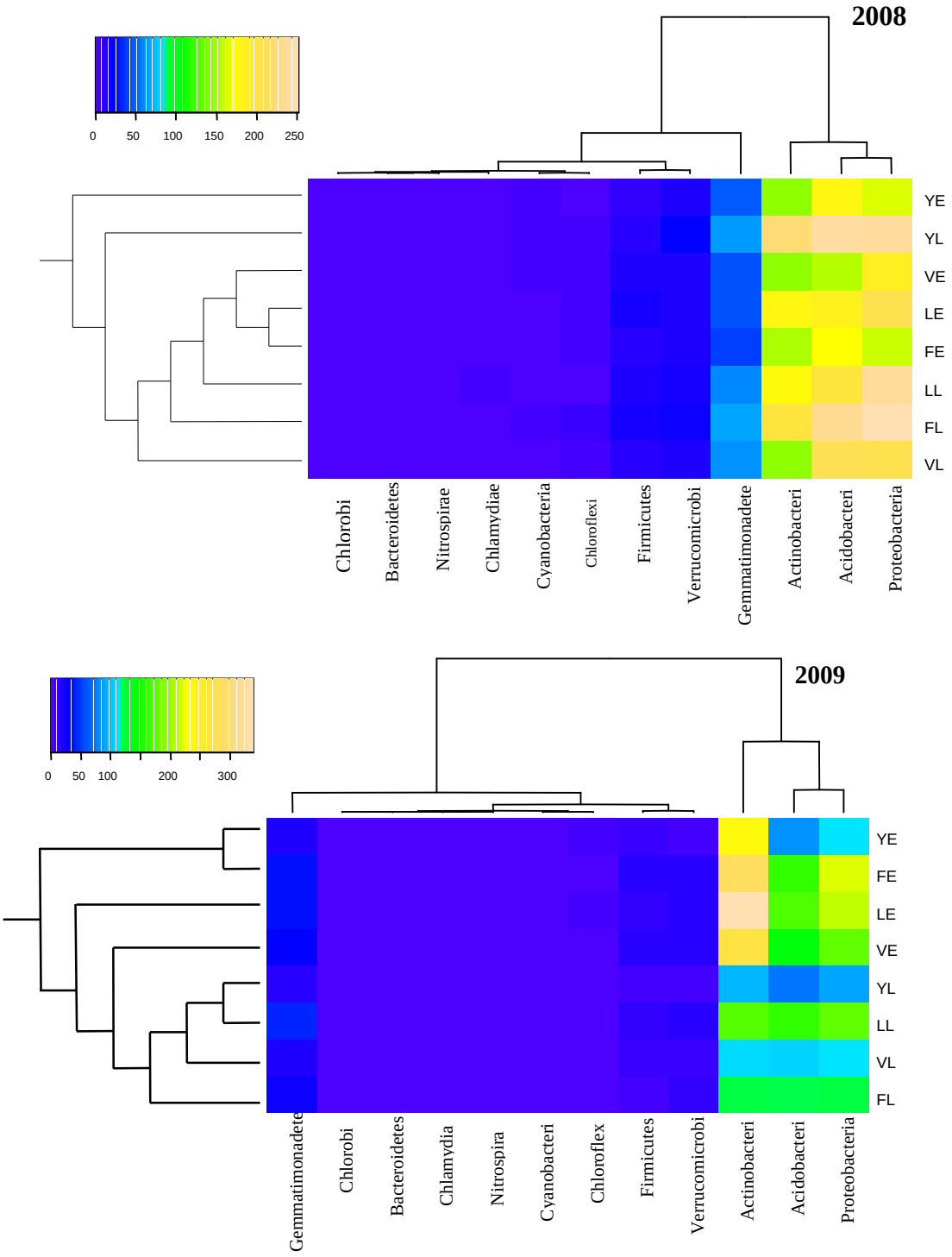

Figure 5.3 Hierarchical cluster analysis of the abundance of bacterial OTUs of different phyla found in the rhizosphere of field-grown pulses in early July (E) and late September (L), in 2008 and 2009. Y, yellow pea; V, chickpea cultivar CDC Vanguard; L, chickpea cultivar CDC Luna; F, chickpea cultivar CDC Frontier.

Hierarchical cluster analysis showed that in both years, the largest differences in rhizobacterial community structure were found between yellow pea plots sampled early July and late September (Figure 5.3), suggesting that plant death changes the rhizosphere environment importantly. By contrast to chickpea, which has an indeterminate growth habit, yellow pea had completed its life cycle in early July. Chickpea cultivars were associated with relatively similar rhizosphere communities in early July, which diverged until late September in 2008 (Figure 5.3), but converged in 2009 (Figure 5.3), suggesting that the effect of drought in summer 2009 had a larger influence on the rhizobacterial community than plant genotypes. The large effect of drought on rhizobacterial communities was also revealed by the decreased bacterial abundance from July to September in 2009, whereas an increase in abundance was seen during the same period in 2008, a wet year.

Diversity indices revealed the absence of a treatment effect in early July 2008 and in the droughty year 2009. In 2008, CDC Luna was associated with the greatest level of rhizobacterial diversity, and yellow pea with the lowest level (Figure 5.5). In September 2008, ANOVA analysis of Chao 1 and ACE richness estimating indices revealed a lower (P = 0.002 and P = 0.001, respectively) bacterial richness in the rhizosphere of yellow pea than that of the three chickpea cultivars (Figure 5). Shannon (H') diversity index revealed the lowest ($P < 0.001$) level of diversity in the rhizobacterial community of the yellow pea in 2008 (Figure 5.5). CDC Luna rhizosphere hosted the most diverse bacterial community of all pulse crops in September 2008 (Figure 5.5). In 2009, the bacterial communities in the rhizosphere of the different pulses had similar Chao 1, ACE, and Shannon diversity indices, further supporting an overriding influence of moisture availability on rhizobacterial communities. The observation of similar rhizobacterial

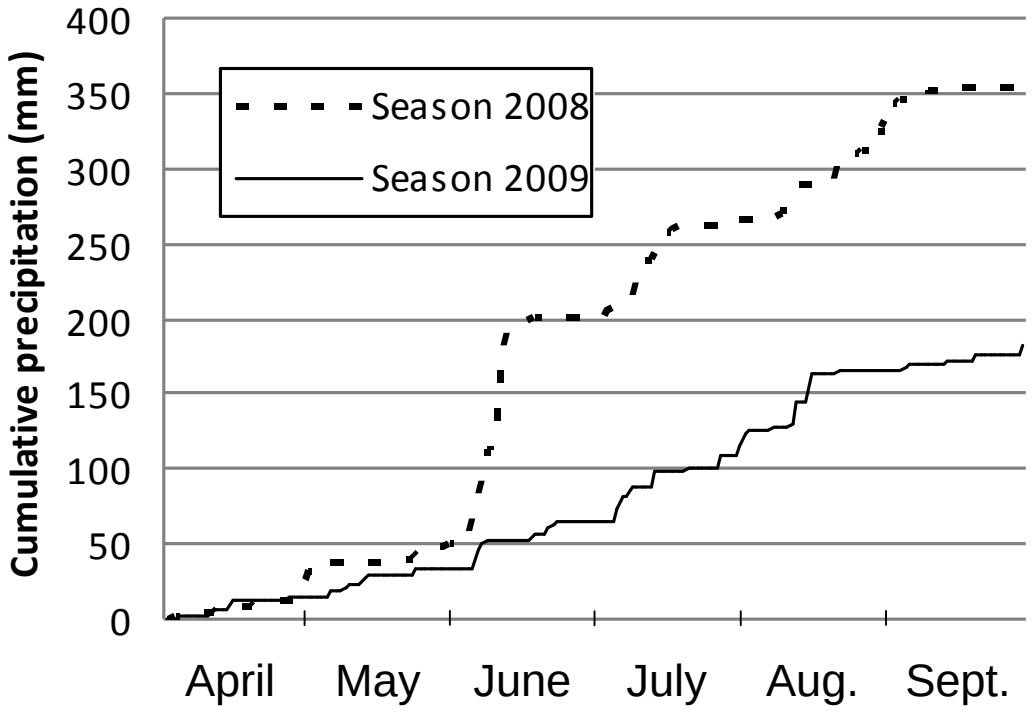

Figure 5.4 Cumulative precipitation from 1 April to 30 September in 2008 and 2009.

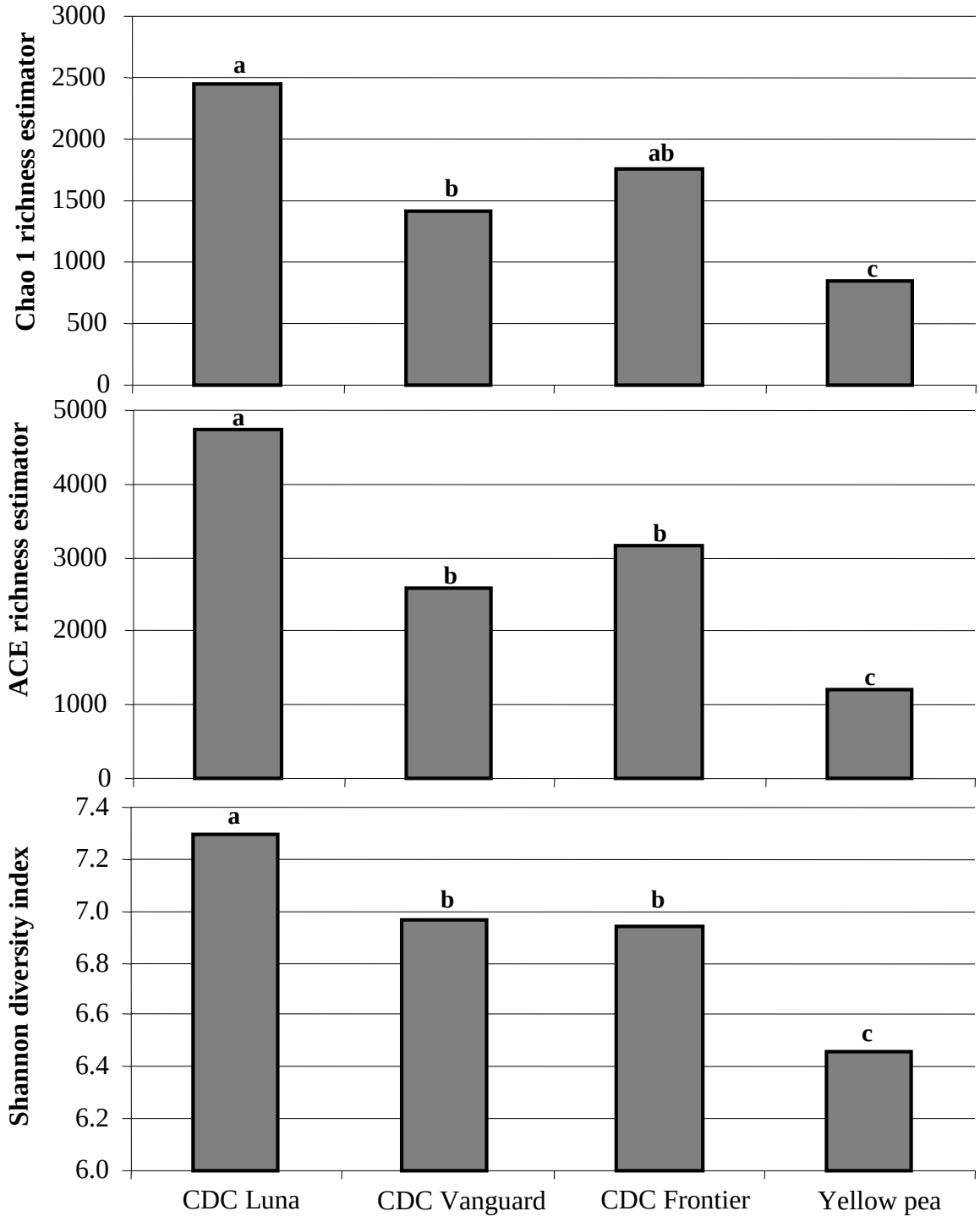

Figure 5.5 Richness estimating indices showed significant differences of rhizobacteria richness of different pulse crops in September of 2008. P value was = 0.002, 0.001 and < 0.001 for Chao 1 richness estimator, ACE richness estimator and Shannon (*H'*) diversity index, respectively. Different small letters means significantly difference base on Fisher-LSD test at $P < 0.05$ (n = 4).

community in all treatments in September 2009 was consistent with the similar functionality of rhizomicrobial communities associated with the different pulse crops examined in the greenhouse assay (Figure 5.2).

5.5 Discussion

Pulse crops bring many benefits to cropping systems (Hayat et al. 2010; Rokhzadi and Toashih 2011) in addition to symbiotic nitrogen fixation (Lindström et al. 2010; Zhao et al. 2010). The positive influence of pulse crops on a following cereal crop reported in the semiarid area of the northern Great Plains of America was attributed to increased soil available N (Badaruddin and Meyer 1994; Beckie and Brandt 1997), enhanced soil water content (Miller et al. 2003a), and reduced cereal disease outbreaks (Stevenson and Van Kessel 1996). The results showed that pulses can provide more benefits to cropping systems, as proposed by others (Bourgeois and Entz 1996; Kirkegaard et al. 2008). It appears that the selective effect of pulse crops on plant-growth-promoting organisms may explain why the beneficial effect of rotation pea on wheat growth could not always be explained by soil N, soil moisture, or disease pressure (Miller et al. 2002).

The pulse rhizosphere environment can select organisms with plant-growth-promotion ability. The different effects of inoculation with rhizosphere soils from different pulses on durum wheat observed in this study indicated that these pulse crops select rhizosphere microorganisms with different growth-promotion potential, and may differentially influence the growth of a subsequent crop.

Roots have a large selective effect on soil microorganisms (Dunfield and Germida 2003; Garbeva et al. 2004), and in particular on plant growth promoting microorganisms (Dutta and Podile 2010). As a result, different crop species (Inceoğlu et al. 2010) or different cultivars of the same species are associated with different rhizosphere

communities (Andreote et al. 2010). Plants exude various organic molecules with potential selective effects. Exuded molecules can be a source of carbon and energy for both growth promoting organisms and competitors, or can modify soil nutrient availability, impacting soil microorganisms. The exuded molecules may also act on bacterial adhesion through pH modification, and contain signal molecules triggering various responses in microorganisms (Dutta and Podile 2010).

Plant growth promoting rhizobacteria can stimulate plant growth by reducing disease pressure, producing growth stimulating compounds such as hormones and enzymes, and by improving plant nutrition in various ways (Hayat et al. 2010). Numerous bacteria isolated from the roots of chickpea and pea in the Canadian Great Plains exhibited plant growth promoting traits (Hynes et al. 2008). The vast majority of these PGPR were Pseudomonadaceae and Enterobacteriaceae of the Proteobacteria, but several were Actinobacteria. This concurs with earlier reports of growth promotion of durum wheat growth by Proteobacteria (Jha and Kumar 2009; Nabti et al. 2010), and Actinobacteria (Hamdali et al. 2008). Proteobacteria and Actinobacteria were two of the three dominant phyla encountered in this study, therefore, the PGPR capability of bacteria belonged to these phyla may due to their dominance in the soil environment (Chauhan et al. 2011; Janssen 2006).

Species of Acidobacteria, the other dominant phylum encountered in this study, are largely unculturable and their function in soil, where they abound, is largely unknown (Dutta and Podile 2003; Janssen 2006). Hunter (2006) showed that the phylum Acidobacteria may also contain species with PGPR activity. I found that the abundance of this phylum was less impacted by drought than Proteobacteria and Actinobacteria, however, plant growth promotion was absent in the greenhouse bioassay in 2009,

suggesting that functions of phylum Acidobacteria can be limited by environmental conditions.

The selective use of plant genotypes with the ability to improve soil biological quality would improve the productivity of cropping systems. I found that good growth promotion is not an ability restricted to yellow pea rhizosphere. Chickpea CDC Luna rhizosphere soil, here, had growth promotion effects comparable to that of yellow pea rhizosphere soil. Thus, reports of the greater value of yellow pea than chickpea, as rotation crops (Miller et al. 2003b), must be considered with caution. It appears that the selection of plant genotypes for their effect on soil biological quality, in addition to their agronomic performance, may bring important advantages to cropping systems.

The variation due to year, which probably reflects the influence of soil moisture availability on pulse rhizobacterial communities, was very large. This is consistent with former studies reporting environmental effects on the diversity and structure of the soil bacterial community (Kennedy et al. 2005; Rasche et al. 2011), which are modulated by the susceptibility of different bacterial groups to drought condition (Welsh et al. 2009). The abundance of precipitation was identified as a main driver of soil bacterial community structure (Meier et al. 2008). This study examined rhizobacterial communities in a wet (2008) and a dry (2009) year and further revealed the importance of precipitation as a modulator of the rotation effect on soil biological quality. When soil water is sufficient, nutrient availability limits rhizobacteria growth (Lugtenberg and Kamilova 2009). However, under drought condition, water becomes the limiting factor directly selecting drought tolerant bacteria (Jin et al. 2011), and indirectly selecting bacteria responding to rhizodeposition of organic compounds by plants due to drought stress (Somasundaram et al. 2009).

Seasonal effects on pulse-associated rhizobacterial community composition were found in this study. The length of the growth period is apparently important in determining the impact of plant genotype on soil bacterial communities (Andreote et al. 2010). A previous study reported that plants secretions differ with growth stages (Meier et al. 2008). In particular, young roots typically excrete more organic materials than older roots (Bowen and Rovira 1991; Lynch and Whipps 1990), providing more energy and nutrients for rhizobacterial growth. Besides, different bacteria have different metabolic strategies to respond to plants (Andrews and Harris 1986), resulting in complex interactions that changes rhizobacterial communities as plants age. At early growth stages or after plant death, r-strategists, which are characterized by fast growth rates but high substrate requirements, will proliferate, increasing the diversity of the rhizobacterial community. While at the late growth stage, K-strategists, characterized by low growth rates but low substrate requirement, will grow better (Andrews and Harris 1986; Zhang et al. 2011). Therefore, the difference of maturing habits among pulse crops modulating seasonal variation in root excretions is an important driver of changes in rhizobacterial community.

5.6 Conclusion

The structure of pulse rhizobacterial community varied with plant genotypes, which may influence the functionality of this community. The rhizosphere community of pulses may promote plant growth and may be a component of the so-called “rotation effect”, which varies in magnitude in different cropping systems. The selection of crop genotypes for their ability to improve soil biological quality may increase the productivity of cropping systems. Environmental factors, such as moisture availability,

however, appeared as a major driver of rhizobacterial community composition, whose influence can override the effect of plant genotype.

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6. Perface

The following chapter is under review in Applied and Environmental Microbiology (Manuscript ID: AEM07433-11). This study showed the effects of different pulse crops on the endophytic bacterial community in durum wheat following pulse crops. The work offered an opportunity to look into the impact of pulse-cereal rotation practices on endophytic bacteria and their potential effects on wheat growth. The work was co-supervised by C. Hamel and V. Vujanovic. Y. T. Gan (co-author) provided his comments to improve this manuscript, and L. Bainard helped to polish the English. I prepared a research plan, handle the sampling, processed samples, analyzed data and prepared this manuscript.

6. ENDOPHYTIC BACTERIA FEEDBACK IN THE CROP ROTATION EFFECTS

6.1 Abstract

A mutual influence exists between plants and their biological environments in agroecosystems, and the legacy of a previous crop on biotic soil properties could feedback on the productivity of the following crop. It is believed that maintenance of plant cover maintains the quality of soil microbial resources. However, plant death and a temporal break in the absence of living plants could favour plant growth promoting microbial groups over plant parasitic groups. Polymerase chain reaction and 454 GS FLX pyrosequencing of amplicons were used to determine the effects of termination time of previous crops on the endophytic bacterial community colonizing the roots of wheat and on the productivity of wheat. In 2008-09, when these contrasting chickpea genotypes (late maturing plants) were terminated as early as pea (July), the microbial legacy of these four previous crops resulted in the establishment of four similar endophytic bacterial communities in wheat roots. These four endophytic bacterial communities were different from those formed following the same chickpea genotypes when crops were terminated late (September). Late terminated crops led to the formation of Firmicutes dominated endophytic bacterial communities, which were less diverse than those Actinobacteria and Proteobacteria dominated endophytic communities formed after early-terminated crops. The Actinobacteria and Proteobacteria dominated

endophytic bacterial communities were associated with altered wheat plant architecture and high grain yield, conversely, the abundance of Firmicutes was associated with low yield. The effect of termination time was weak in 2009-2010, and probably overriden by the effect of abnormaly high precipitation received during this period. The results showed that a temporal break in plant cover can improve the microbial quality of the agroecosystem.

6.2 Introduction

Crop rotations have supported human societies through history (Anderson 2005), as far back as the Roman empire (Karlen et al. 1994). In particular, pulse-cereal rotations were traditionally used for their positive influence on soil biological quality and plant health as it can break disease cycles (Karlen et al. 1994), optimize soil water use efficiency (Pala et al. 2007), improve soil aggregation (Masri and Ryan 2006), increase soil available N (Pierce and Rice 1988), soil organic matter (Ryan et al. 2008b), and crop yield (Ryan et al. 2010).

Plants have evolved with the capacity to modify their soil microbial environment with feedback on the productivity of plant community (Bever 2003; Van de Voorde et al. 2011). Plant roots strongly influence soil microorganisms, providing them with niches and nutrients (Bowen and Rovira 1991). Some bacteria associated with plant roots are capable of living inside the plant tissue without causing plant disease (Sturz et al. 2000). Although endophytic bacteria occur at low population densities in roots (Rosenblueth et al. 2004), they may stimulate plant growth (Rosenblueth and Martínez-Romero 2006). In wheat (*Triticum aestivum* L.), endophytic bacteria from different phyla were reported (Coombs and Franco 2003; Iniguez et al. 2004). However, the effect of the bacterial colonization on plant growth remains poorly understood (Meier et al. 2008).

Chickpea (*Cicer arietinum* L.) is planted in arid and semiarid regions worldwide (Kyei-Boahen et al. 2002; Millan et al. 2006; Pande et al. 2005) often in rotation with wheat. Chickpea has an indeterminate growth habit (Anbessa et al. 2007). It can use water from deeper soil layers and grow vegetatively well into fall (Gan et al. 2008) in contrast to pea which matures as early as July. Previous research has reported that plants at different growth stages select rhizobacterial communities (Bowen and Rovira 1991) with different metabolisms (Andrews and Harris 1986), and different symbiotic relationships with their host plants (Roesti et al. 2006). I hypothesized that the later termination of chickpea could select a rhizobacterial community with reduced growth promoting abilities, resulting in lower productivity of durum wheat grown after chickpea than after pea. The objective of this study was to: 1) describe the effects of termination time of previous pulse crops on the endophytic bacterial community colonizing the roots of durum wheat grown the following year, and 2) to explore the relationship between the structure of endophytic bacterial communities and wheat yield under field condition.

6.3 Materials and methods

6.3.1 Experimental design and treatment application

A field experiment was set out in a randomized complete block design with four blocks at the South Farm of the Semiarid Prairie Agricultural Research Centre (SPARC) in Swift Current, SK, Canada (latitude 50° 18’ N; longitude 107° 41’ W). The experiment was conducted in 2 m × 8 m plots in 2008-2009, and repeated in 2009-2010. Seven preceding pulse crop treatments were applied at stage-1 of a 2-year crop rotation series with durum wheat (*Triticum turgidum* var *durum* Desf.) cultivar AC Avonlea at stage-2. They were: an early maturing yellow pea crop (cultivar CDC Handel [Y]), three chickpea cultivars terminated as early as the yellow pea by mowing (CDC

Vanguard [VE], CDC Luna [LE], CDC Frontier [FE]), and the same three chickpea cultivars terminated late (CDC Vanguard [VL], CDC Luna [LL], CDC Frontier [FL]) i.e., when they reached full maturity (Table 6.1). Durum wheat was planted at stage-2 of the rotation at a seeding rate of 113 kg ha^{-1}. Durum wheat was fertilized with 43 kg ha^{-1} of P (11-51-0) and 111 kg ha^{-1} of N (46-0-0). Roundup WeatherMAX® was applied at 815 ml ha^{-1} on May 5th and Achieve® Liquid Gold was applied at 490 ml ha^{-1} on June 3rd for weed control.

6.3.2 Root sampling

Durum wheat root samples were taken at flag-leaf stage. Five plants were randomly taken from five locations in each plot using a shovel. Shoots were detached, and roots were placed in plastic bags and kept at 4°C for a few hours until processing. Root samples were washed under running tap water and cut into 1-cm fragments. A representative subsample (2 g fresh weight) from each plot was placed into a 1.5-ml plastic tube, surface sterilized for 1 min in 70% ethanol mixed with 30% hydrogen peroxide (1:1), and rinsed several times with sterile distilled water. Cleaned root samples were oven dried at 50 °C for 24 h and finely ground in a bead miller (Retsch, MM301).

6.3.3 Characterization of endophytic bacterial communities

Raw DNA was extracted from ground root samples using a DNeasy Plant Mini Kit (QIAGEN group, Toronto, ON, Canada) following the manufacturer's protocol. After diluting 10 times, DNA was subjected to polymerase chain reaction (PCR) using primers 968f / 1401b amplifying bacterial 16S-rDNA universal gene fragments (Watanabe et al. 2001). 454 Life Science's A or B sequencing adaptors were fused to the 5' end of forward or reverse primers, and unique barcode sequences were added between the A

Table 6.1 Description of the genotype / termination time treatments applied at rotation phase-1 to measure their effects on the durum crop grown in rotation phase-2.

	Rotation phase-1			Rotation phase-2
Treatment	Pulse genotype	Termination time		
Y	Yellow Pea	Early July	-	Durum wheat
VE	CDC Vanguard	Early July	/	Durum wheat
LE	CDC Luna	Early July	/	Durum wheat
FE	CDC Frontier	Early July	/	Durum wheat
VL	CDC Vanguard	/	Late September	Durum wheat
LL	CDC Luna	/	Late September	Durum wheat
FL	CDC Frontier	/	Late September	Durum wheat

adaptor and the forward primer in order to trace the sources of sequences after multiplex sequencing of amplicons (as shown in Table 3.2 of Chapter 3).

Platinum® PCR SuperMix (Cat. No. 11306-016, Invitrogen™) was used for PCR. Thermal cycling was conducted in an Veriti™ 96-well fast Thermal Cycler (Applied Biosystems) with the following conditions: 4 min initial denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 56 °C and 1 min elongation at 72 °C; and a 15 min final elongation at 72 °C.

All PCR products were purified on agarose gel. Briefly, PCR products were run in 1% (w / v) agarose gel under 65 V for 1 h, then, gel pieces containing visible bands of target size were cut off with a sterile blade, put into a sterile centrifuge tube with 30 µl TE buffer (1×dilution), vortexed for 1 min and placed at 4 °C over night for extraction. The concentration of purified PCR products in TE buffer was measured with a Nano Drop-1000 spectrophotometer (Thermo scientific®). The concentration of each sample was adjusted to 30 ng μl^{-1}. PCR products carrying different MIDs were placed in a sterile 1.5 ml plastic tube in pools of 16, and submitted for pyrosequencing at Génome Québec (Montréal, QC, Canada).

6.3.4 Head number, grain yield and grain protein of durum wheat

At physiological maturity, one 1-m^2 quadrat of durum plants was randomly selected from each plot, and all the heads of the plant in the quadrat were counted. At full maturity, durum wheat was harvested and the seeds from each plot were cleaned to remove debris, oven dried, and dry weights were recorded. A 350-g seed sample from each plot was used for grain protein determination using an Infratec 1229 Grain Analyzer (Foss Tecator, AB).

6.3.5 Bioinformatic and data analysis

All sequences were edited to remove primers, MID, and adaptor sequence fragments, using Mothur V.1.15.0 (Schloss et al. 2009). All sequences used in this study shared at least 97% similarity with known sequences (Wu et al. 2010a), based on the Silva database (http://www.arb-silva.de/). Rarefaction analysis, classification of dominant phyla and heat map analysis were also conducted using Mothur V.1.15.0. Effects of experimental treatments on the Chao 1 and ACE richness estimating indicators were determined using Mothur V.1.15.0. The effect on durum wheat yield was tested by ANOVA in SYSTAT 12.0, and the significant differences between treatments were tested with Fisher-LSD test at the 5% level. The significance of termination time effect on the proportion of dominant bacterial phyla in wheat roots was assessed by MultiResponse Permutation Procedure (MRPP) in PC-ORD. Effects of treatments on crop-related response variables were detected by Multivariate analysis of variance (MANOVA) of SYSTAT 12.0. The relationship between crop-related response variables (durum wheat heads m^{-2}, percentage of grain protein, grain yield) and bacterial community structure, described as the number of OTUs measured in each bacterial phyla, was assessed by redundancy analysis (RDA) (Borcard 2011) and plotted using R (R Core Development Team 2009) with the package Vegan 1.15-4 (Dixon 2003).

6.4 Results

Precipitation in 2008-2009 was lower than the long-term average, however, in 2009-2010 precipitation was much higher than normal especially during the period from May to July (Figure 6.1). The large variation in precipitation between two test years may have influenced the soil environment and microbial communities.

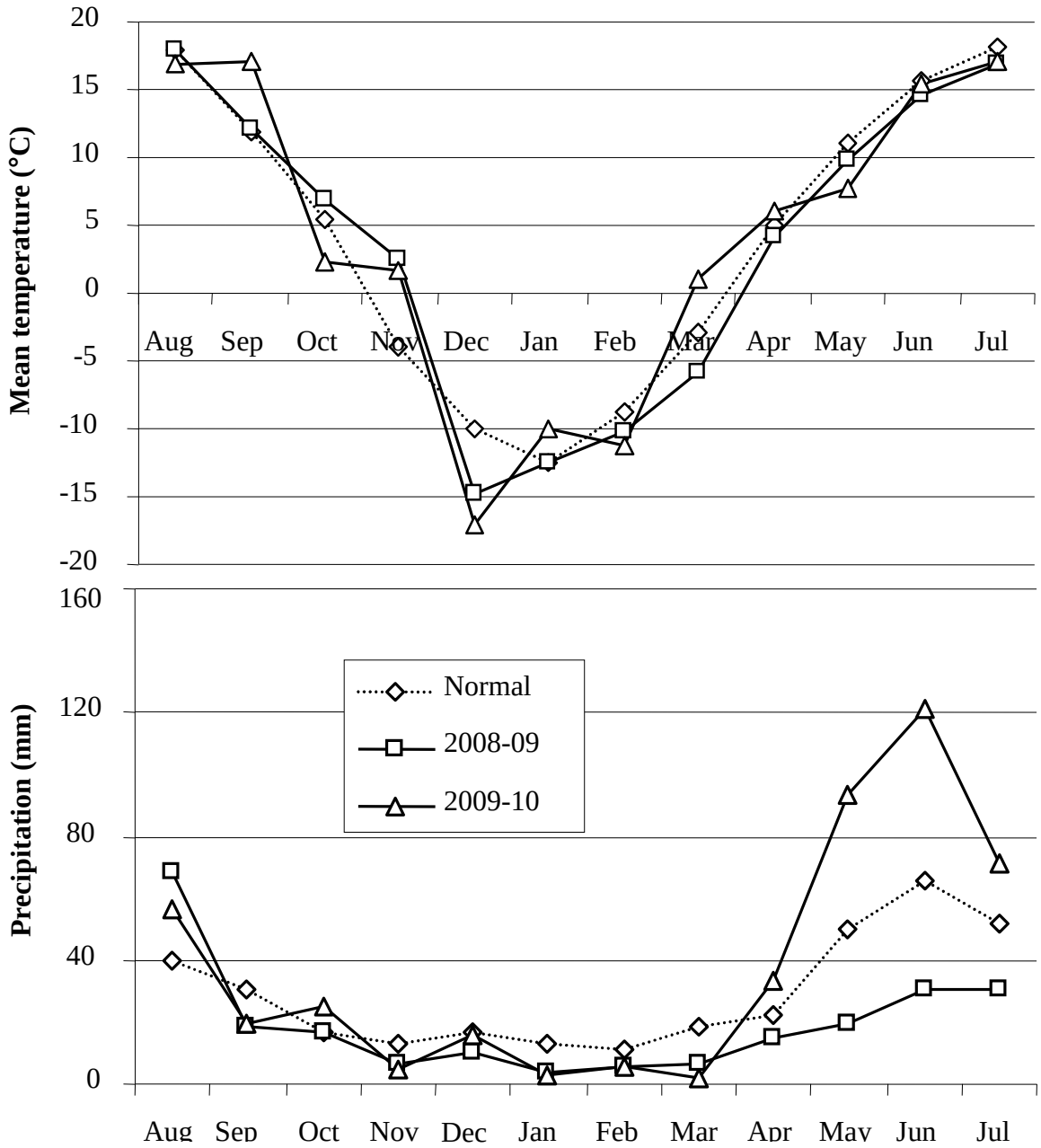

Figure 6.1 Temperature and precipitation recorded in 2008-2009 and 2009-2010, as compared to normal (1971-2000, Enviroment Canada).

6.4.1 Effect of pulse termination time on bacterial communities in durum wheat roots

Overall, the endophytic bacterial richness in durum wheat roots was higher in 2009 than in 2010 (Figure 6.2). ANOVA detected a significant effect of preceding pulse crops on bacterial community richness in durum wheat roots in 2009 (Table 6.2). Endophytic bacteria richness was lower in the roots of durum wheat following late-terminated pulse crops in 2009 compared with early-terminated pulses.

The richness of the endophytic bacterial community following pea did not differ from that following the early-terminated chickpea crops. However, in 2010 the preceding pulse crops did not influence the richness of the endophytic bacterial community in the succeeding durum wheat.

Heat map analysis of the structure of the endophytic bacterial communities, assessed as the abundance of OTUs distributed in different phyla, also revealed differences between termination times (Figure 6.3). In 2009, high similarity was found in communities inhabiting durum wheat roots following early-terminated pulse crops as shown by high Yue & Clayton Theta similarity coefficient (light color). Similarity was also high in communities following late-terminated pulse crops, but less similarity was found between endophytic bacterial communities following early-terminated and late-terminated pulses. These results suggest that different pulse termination times at phase-1 of the crop rotation affects the composition of durum wheat endophytic bacteria communities. MRPP analysis conducted on the three dominant bacterial phyla of these communities, Actinobacteria, Firmicutes and Proteobacteria, confirmed the high significance ($P < 0.001$) of two termination times on the endophytic bacterial community structure of durum wheat roots. Higher proportions of Actinobacteria and

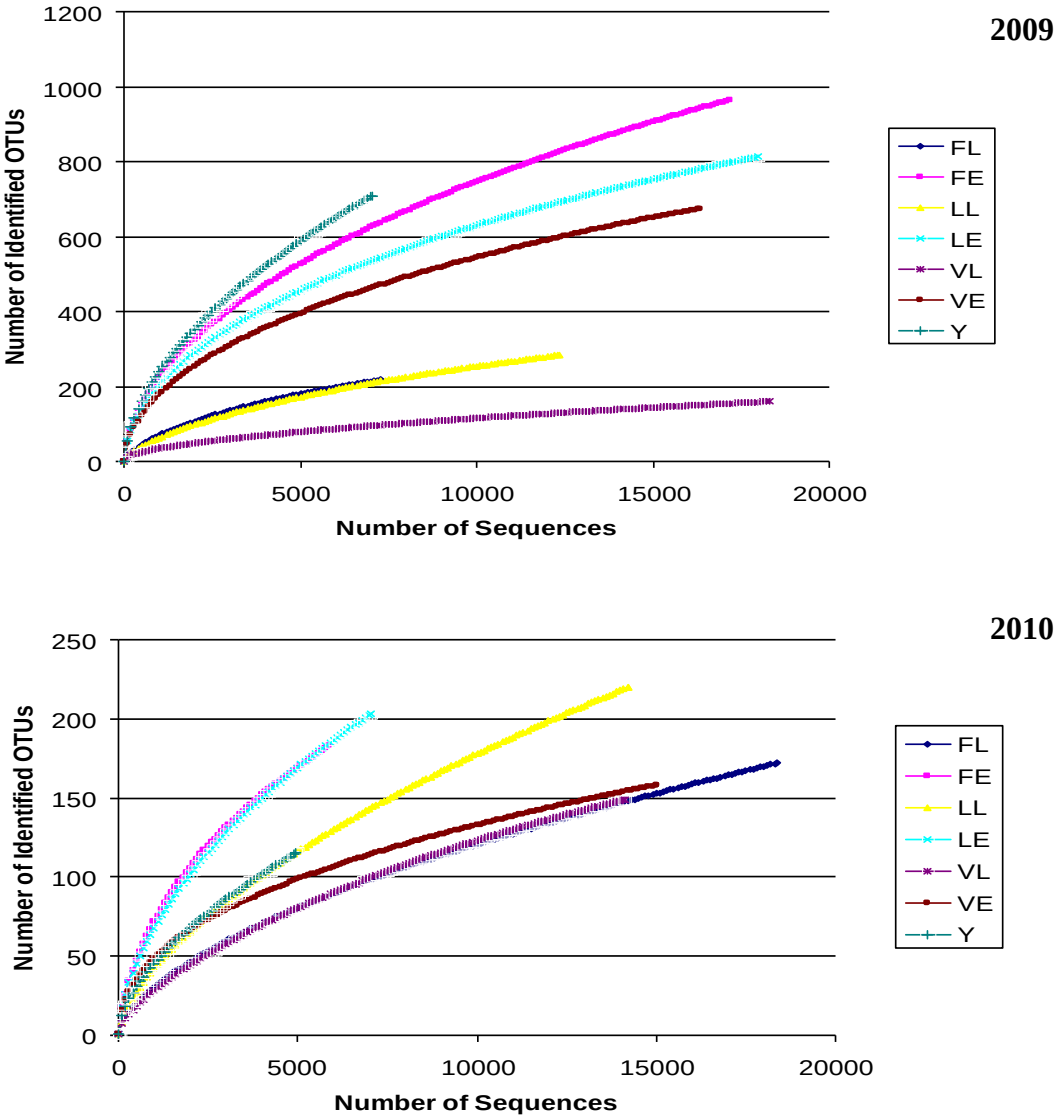

Figure 6.2 Rarefaction curves showing the relationship between sequencing depth and wheat root OTUs richness for each treatment in 2009 and 2010. OTUs were calculated based on 97% similarity with known sequences from Genebank. FL: Late terminated CDC Frontier; FE: Early terminated CDC Frontier; LL: Late terminated CDC Luna; LE: Early terminated CDC Luna; VL: Late terminated CDC Vanguard; VE: Early terminated CDC Vanguard; Y: Yellow pea (Early maturity).

Table 6.2 Effects of different preceding pulse crops on the richness of the endophytic bacterial community of durum wheat roots in 2009 and 2010.

Experimental year		2009		2010	
Richness estimator		Chao 1‡	ACE	Chao 1	ACE
Pulse crops	Y	721 ab	1022 a	111	160
	LE	687 b	869 ab	192	367
	VE	748 ab	783 b	171	254
	FE	950 a	1229 a	151	191
	LL	277 c	374 c	239	310
	VL	155 c	223 c	188	273
	FL	217 c	329 c	230	375
	P value	<0.0001	0.001	0.465	0.227

Note: ‡Means associated with different letters within a column are significantly different at $P < 0.05$, according to one-way ANOVA; $n = 4$.

FL: Late terminated CDC Frontier; FE: Early terminated CDC Frontier; LL: Late terminated CDC Luna; LE: Early terminated CDC Luna; VL: Late terminated CDC Vanguard; VE: Early terminated CDC Vanguard; Y: Yellow pea (Early maturity).

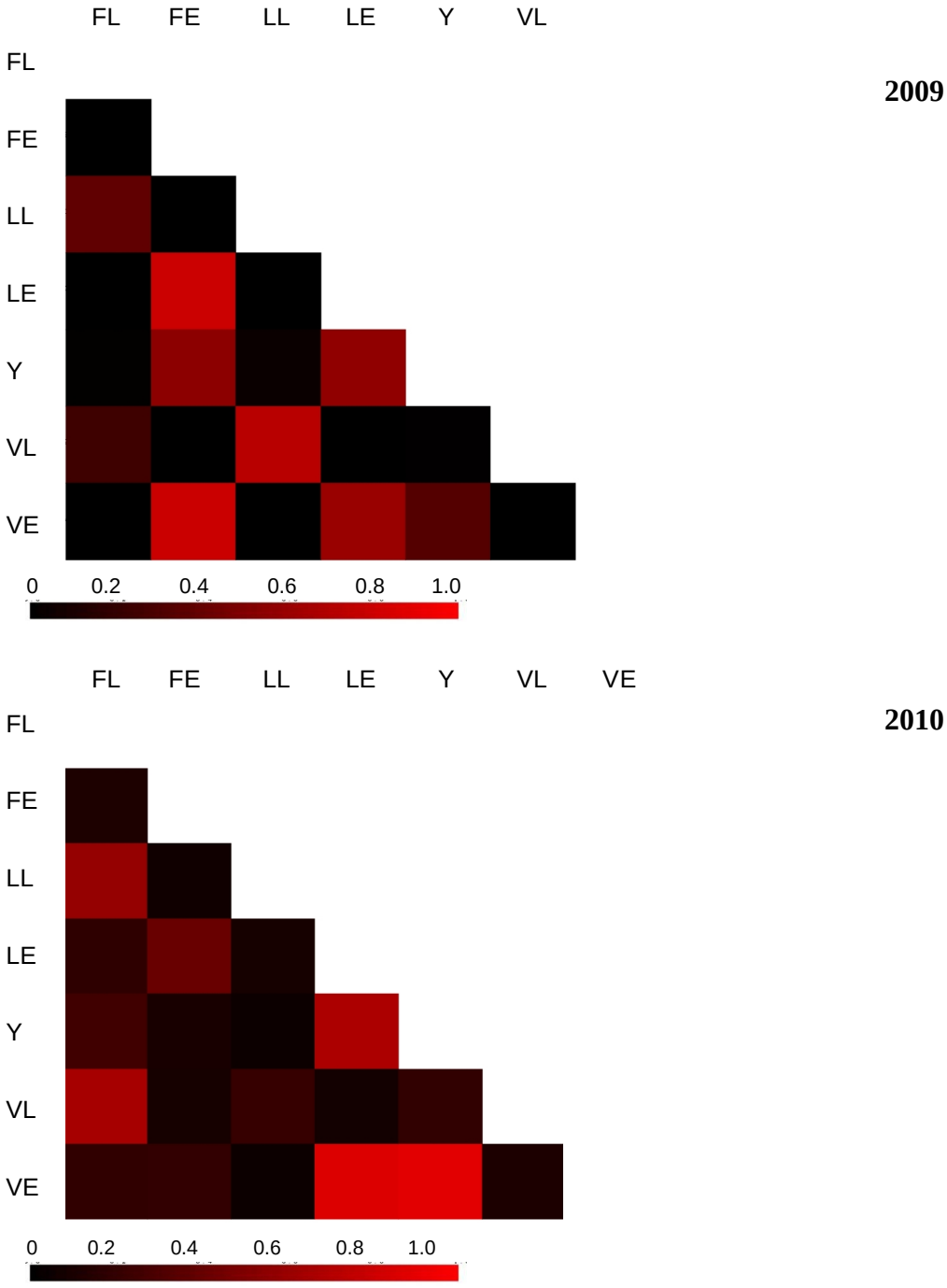

Figure 6.3 Heat map analysis at 97% similarity based on Yue & Clayton Theta coefficient of similarity calculated based on the bacterial OTUs profile found in the roots of durum wheat, as influenced by the termination time and genotype of a previous pulse crop in 2009 and 2010.

Proteobacteria were found in durum wheat roots after the early-termination of pulse crops, while Firmicutes dominated after a late-terminated pulse crop (Figure 6.4). The Actinobacteria, which comprised 42% - 65% of total identified OTUs in durum wheat roots following an early-terminated pulse crop, made up less than 5% of total identified OTUs following a late-terminated crop, where Firmicutes was dominant and accounted for more than 80% of total identified OTUs.

6.4.2 Durum wheat yield

ANOVA showed the effect of termination time on durum wheat grain yield in 2009 was significant ($P < 0.001$). Grain yield of durum wheat was lower after a late-terminated pulse crop than after an early-terminated pulse crop (Figure 6.5). RDA results showed that endophytic bacterial community structure was correlated ($P = 0.001$) with durum wheat grain yield and with the number of heads per m^2 (Figure 6.6). A negative relation between grain yield and abundance of heads suggests an early influence of endophytic bacteria on tiller formation leading to a lower number of reproductive stems bearing larger spikes, where early pulse termination increased durum wheat yield. The abundance of Firmicutes, the phylum dominating in durum roots after late pulse crop termination, was negatively related with wheat yield, and positively related with the number of heads per m^2, suggesting a role for some endophytic bacteria in the modification of durum wheat plant development. The percentage of protein in the grain was unrelated to endophytic bacteria colonization of durum wheat roots (Figure 6.6).

6.5 Discussion

The feedback mechanism of soil microbial communities on plant growth (Bever 2003; Van Der Heijden 2002) generates significant interest in plant ecology. How plants influence their community through their effects on the soil biota is important in

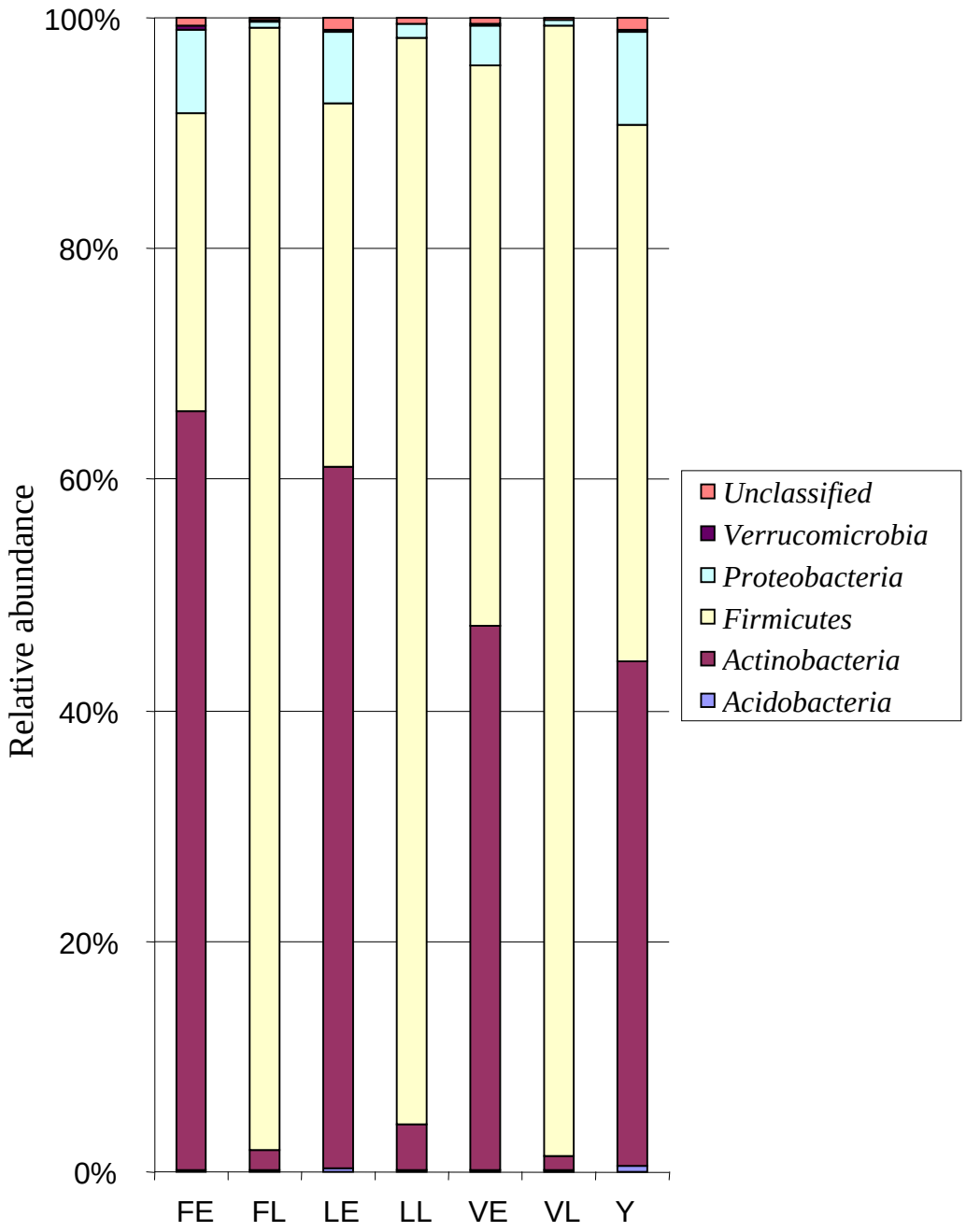

Figure 6.4 Relative abundance of endophytic bacterial phyla found in the roots of durum wheat grown in 2009, as influenced by the time of termination of previous pulse crops.

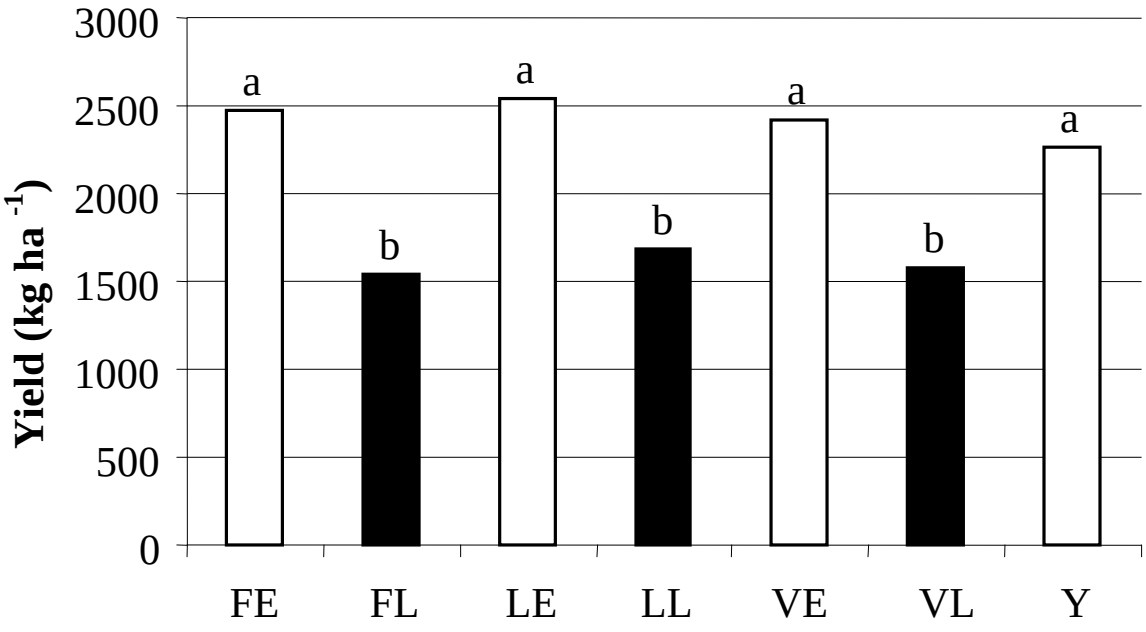

Figure 6.5 Durum wheat grain yield measured in 2009, as influenced by the termination time and genotype of a pulse crop grown in 2008. ($P < 0.001$, $N = 28$).

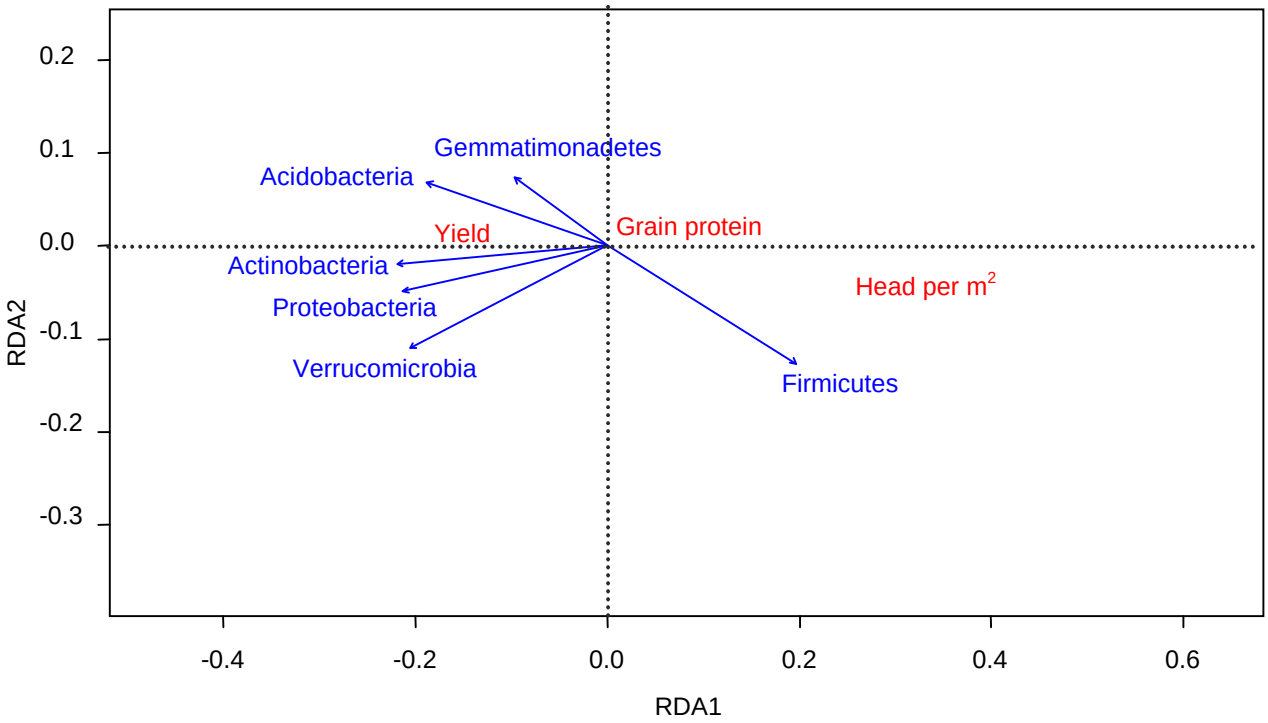

Figure 6.6 Redundancy analysis (RDA) showing the relationship among identified endophytic bacterial phylum, number of wheat head per m^2, grain protein and yield of durum wheat in 2009 (P = 0.001, N = 28).

explaining the dynamics of plant community composition (Kulmatiski and Beard 2011; Peh et al. 2011; Reinhart et al. 2003) and the process of plant invasiveness (Lankau 2011; Reinhart and Callaway 2006). The concept of soil community feedback has also been applied to field crops (Hamel et al. 2005).

The results of the present study showed the effects of soil microbial community on durum wheat growth in crop rotation. It is very difficult to disentangle the different influences making up a "rotation effect" (Kirkegaard et al. 2008). This is particularly true in the case of the rotation effect of chickpea. As compared with other pulse crops such as yellow pea, chickpea is less beneficial to the productivity of a following wheat crop (Miller et al. 2003). Whereas differences can be attributed to different water use and biological nitrogen fixation legacy in the different pulse crops (Miller et al. 2003; Miller et al. 2002), a large part of the difference remains unexplained (Bourgeois and Entz 1996). Varied composition of cereal rhizobacterial communities found in different pulse-cereal rotation series (Alvey et al. 2003), suggested that different pulse crops impact the rhizobacterial community of a following cereal crop differently. The present study showed that the influence of contrasting crop termination time on the soil microbiota largely explained the poor rotation effect of chickpea. This conclusion is supported by studies showing little immediate effect of the plant itself on the soil microbiota in field situation (Kulmatiski and Beard 2011).

Change in the soil microbiota following the establishment of a new crop was shown to proceed slowly over a few years (Hamel et al. 2005). By contrast, decomposing plant residues in soil initiates within hours, which is a rapid succession of microorganisms with increasing ability to decompose complex organic compounds (Astaraei 2008; McMahon et al. 2005). Changes in the soil microbiota induced by decomposing residues

may feedback on crop yield, as observed in this study. Different root endophytes, which are known to influence plant growth, are subsets of the soil microbiota (Rosenblueth and Martínez-Romero 2006). Consequently, changes in the bacterial community structure in durum wheat roots with changes in preceeding pulse crop termination time is likely attributable to variation in the abundance of key microorganisms in the microbial pool under the influence of decomposing residues.

The abundance of Actinobacteria was related with high durum wheat yield in this study. The number of Actinomycetes, a diverse group of Gram + actinobacteria able to depolimerize recalcitrant compounds (Paul and Clark 1996), often peaks in the last stage of microbial succession in decomposing plant residues (Astaraei 2008). In this study, the abundant Actinobacteria in the seedbed of durum wheat after an early-terminated pulse crop may have resulted in abundant colonization of durum roots by endophytic Actinomycetes with biocontrol activity. Endophytic *Streptomyces* (Actinomycetes) isolated from wheat roots were found to be effective in antagonizing the pathogenic fungus *Gaeumannomyces graminis* (Coombs et al. 2004), an important pathogen of wheat in Saskatchewan (Bailey et al. 2001). The abundance of Actinobacteria in durum roots after early pulse termination may be the cause of the better durum wheat yield observed. Over 11% of the 116 isolates with plant growth promotion properties found in pulse crops in Saskatchewan were Actinobacteria (Hynes et al. 2008).

A positive feedback of the soil microbiota on durum wheat yield, following an early-terminated pulse crop, could be derived from the abundance of certain plant growth promoting Proteobacteria or Actinobacteria, two bacterial groups associated with early pulse termination and high durum wheat yield in this study. Endophytic Proteobacteria and Actinobacteria were reported in wheat (Conn and Franco 2004; Iniguez et al. 2004)

and related to important functions including increasing drought tolerance and nutrient uptake in host plants (Arzanesh et al. 2011; Hamdali et al. 2008; Jha and Kumar 2009; Nabti et al. 2010).

Plant growth promotion ability of certain Firmicutes was also reported (Rosenblueth and Martínez-Romero 2006), but this bacterial phylum contains several plant pathogens as well. In particular, Phytoplasma, a group of specialised Firmicutes, are obligate parasites of plant phloem tissue causing several complex disease syndromes with symptoms such as stunting, excessive branching, formation of sterile-deformed flowers, virescence, growth reduction, smaller leaves, and phyllody in many plant species (Lee et al. 2000; Olivier et al. 2009). Phytoplasma were reported as the causal agent of wheat blue dwarf disease, a very important disease of wheat in arid and semiarid areas (Wu et al. 2010b). Phytoplasma infection of wheat in Saskatchewan and Manitoba led to malformed seeds and grain yield reduction (Olivier et al. 2009). A stimulating influence of late chickpea termination on parasitic Firmicutes proliferation in durum wheat roots may have resulted in a negative feedback of the soil microbiota on durum wheat productivity. It is also possible that late-terminated chickpea has a neutral influence on the following durum wheat, but the soil microflora associated with pea may have a positive effect on durum wheat, which created the difference in the productivity of durum wheat following chickpea and pea.

Endophytic bacteria are common in plants, as plants provide diverse niches for many kinds of endophytic organisms (Sturz et al. 2000). These organisms also benefit their host plants, as several plant growth promoting endophytic bacteria belonging to *Pseudomonadaceae* and *Enterobacteriaceae* were reported in several pulse crops (Hynes et al. 2008). The abundance of endophytic species in host tissues can vary with time

(Conn and Franco 2004; Kuklinsky-Sobral et al. 2004) and it cannot be ruled out that a change in the bacterial endophyte community composition in chickpea roots from July to September, rather than the impact of crop residues decomposition, is one of the main causes of reduced productivity in the following durum wheat.

The absence of termination time effect might be due to weather in 2009-10, where the extremely wet condition reduced or masked potential treatment effects. Increased soil water content can directly affect the physiological status and symbiotic capability of soil bacteria. Soil moisture is a key factor in soil organic matter decomposition, influencing gas diffusion rate, soil temperature, soil pH, the osmotic status of soil bacterial cells, and substrate availability (Griffiths et al. 2003; Harris 1981). High soil water content can also impact the bacterial endophyte community in cereals by influencing plant growth, root health and rhizodeposition (Lynch and Whipps 1990). Unusual moisture level in 2010 was probably the dominant process selecting soil bacterial communities. The strength of the process was seemingly overwhelming the plant-related selective processes. The significant influence of unpredictable climate reduces our ability to precisely manage agroecosystems.

6.6 Conclusion

The termination time of pulse crops had a significant influence on the composition of bacterial endophyte communities living in the roots of the subsequent durum wheat crop. The modification of the microbiota by pulse rotation crops feedback on the productivity of the wheat crop following the pulses. The declining quality of organic residues as a substrate for soil microorganisms with time since the termination of a pulse crop most likely promoted the selection of beneficial bacterial endophyte, after an early-terminated crop. The effects of different pulse crop termination times on durum wheat bacterial

endophyte communities and productivity is seemingly sensitive to environmental conditions, such as soil moisture level.

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7. Preface

Novel plant growth promoting bacteria called H_2-oxidizing bacteria was identified in this study. Although no fungicide treatment or chickpea genotype effects on H_2-oxidizing bacteria were detected in this study, the identification of this spedific functional bacterial group offered an opportunity to review the existence of H_2-oxidizing bacteria and their influence on host plant growth in chickpea field, which was reported in the following chapter. This study demonstrated that chickpea growth can be modified by the inoculant of H_2-oxidizing bacteria, which may benefit to both chickpea and following cereal crops in pulse-cereal rotations.

The work was co-supervised by C. Hamel and V. Vujanovic. K. Hanson provided assistance with set up of mixed gas incubation system. I prepared a research proposal, planed sampling, processed and analysed samples, cultured and collected clones, and interpreted the data.

7. HYDROGEN-OXIDIZING BACTERIA WITH POSITIVE EFFECTS ON PLANT GROWTH ISOLATED FROM CHICKPEA FIELD

7.1 Abstract

Some H_2-oxidizing bacteria associated with N_2-fixing soybean grown under subhumid climate were shown to promote plant growth. In this study, selective-medium based culture method and molecular methods (polymerase chain reaction – Sanger sequencing technology) were used to isolate and identify H_2-oxidizing bacteria from the rhizosphere of two chickpea (*Cicer arietinum* L.) cultivars growing in semiarid Saskatchewan, Canada. The plant growth-promoting effect of identified bacterial isolates was tested under both laboratory and greenhouse conditions, in a randomized complete block design experiments with five replicates. A total of 1286 bacterial clones were isolated and five different positive H_2-oxidizing bacteria were detected by the Methylene Blue assay. Among these, two strains of *Variovorax paradoxus* and one strain of *Rhodococcus erythropolis* showed significant plant growth promoting effects on durum wheat (*Triticum turgidum* var *durum* Desf.) root elongation and chickpea growth in bioassay. Therefore, I reported an important H_2-oxidizing bacteria presence in chickpea rhizosphere under semiarid condition, and the results also showed H_2-oxidizing bacteria associated with chickpea have the potential to benefit both the durum wheat and chickpea components in pulse-cereal rotation.

7.2 Introduction

Legumes have long known positive effects on the productivity of cereal-based cropping systems (Peoples and Craswell 1992). Among all possible benefits of legumes, biological nitrogen fixation is certainly the most important one (Rochester et al. 2001). However, nitrogen fixation does not satisfactorily explain all improvements in soil structure (Rochester et al. 2001), water-holding capacity (Peoples and Craswell 1992), nutrient availability (Nuruzzaman et al. 2005) and plant health (Stevenson and Van Kessel 1996a) brought about by legume plants in crop rotation. Among these other benefits from legume, "hydrogen fertilization" of soils was reported (Dong et al. 2003).

Hydrogen gas is a by-product of nitrogen fixation. Its release from soybean nodules accounts for about 35% of the energy consumed in nitrogenase activity (Hunt and Layzell 1993). Some Rhizobium species (H_2-oxidizing bacteria) possess the enzyme hydrogenase and can recycle the hydrogen gas released during nitrogen fixation, reducing the amount of energy lost. However, hydrogen uptake ability is rare and most rhizobia lack this ability (Baginsky et al. 2002). Under field condition, the amount of hydrogen gas produced by nodules and released into the soil system can be as high as 240,000 L per hectare during a growing season in a soybean crop fixing about 200 kg N ha^{-1} (Dong et al. 2003). Although hydrogen gas production rate is high and the hydrogen gas release from soil into the atmosphere is expected to be large (Conrad and Seiler 1979a), very little hydrogen gas fluxes from the soil surface can be measured (Conrad and Seiler 1979b), indicating that hydrogen is being used in the soil system.

Previous studies have shown that hydrogen uptake is associated with CO_2 fixation (Stein et al. 2005), soil microbial growth (Popelier et al. 1985) and shift in the structure of the soil microbial community (Stein et al. 2005). Furthermore, the plant growth

promoting effects of hydrogen released in soil was demonstrated under both greenhouse and field conditions (Dong et al. 2003), and attributed to a response of certain bacteria which are able to grow profusely using hydrogen gas as an energy source (McLearn and Dong 2002). However, the isolation of H_2-oxidizing bacteria was not always successful (Haring et al. 1994), and the limited H_2-oxidizing bacteria obtained so far were soybean (*Glycine max* L. *Merr*.) associates from subhumid climates (Maimaiti et al. 2007). To the best of my knowledge, the H_2-oxidizing bacterial association was not studied in chickpea (*Cicer arietinum* L.), or in semiarid conditions.

Chickpea is a dryland crop, requiring abundant fungicide application (Gan et al. 2006), which may adversely affect agriculturally important microorganisms, including H_2-oxidizing bacteria, and reduce the performance of the agroecosystems (Gaind et al. 2007). A better understanding of the beneficial microbial resources available in cultivated soil will lead to the design of sustainable cropping system. In this study, I tested two hypothesis: 1) H_2-oxidizing bacteria exist in chickpea fields under semiarid climate, and 2) these H_2-oxidizing bacteria can stimulate plants growth.

7.3 Materials and methods

7.3.1 Experimental field and soil sampling

Bacteria were recovered from a chickpea field experiment in Swift Current, SK, Canada (latitude 50° 18’ N; longitude 107° 41’ W) in 2008 and 2009, which contained 64 plots in total. Field soil contained 3.60 $kg.ha^{-1}$ N, 21.81 $kg.ha^{-1}$ P, and 283 $kg.ha^{-1}$ K in 2008, and 3.06 $kg.ha^{-1}$ N, 12.58 $kg.ha^{-1}$ P, and 210 $kg.ha^{-1}$ K in 2009. Rhizosphere soil samples were taken from chickpea plots at early flower stage. The first cm of the soil surface was removed to eliminate plant debris, five plants were dug from each plot using a shovel, bulk soil was gently shaken off and plant roots with adhering soil were pooled

to yield one composite sample per plot. Samples were put on ice and taken to the laboratory, root adhering soil was carefully brushed down and sieved through 2 mm. Soil samples were stored in sealed plastic bags at -20°C for further analysis.

7.3.2 Isolation of H_2-oxidizing bacteria

The isolation procedure used in this study followed the protocol of Maimaiti (2007) with few modifications. Soil samples collected from chickpea field were incubated at 22°C in an atmosphere of air and H_2 (1:4) for 21 days in sealed plastic bags (Dong and Layzell 2001). After 21 days of incubation, 1 gram of each soil sample was picked up and serially diluted (10^{-5} ~ 10^{-8}) with sterile water. 1 ml of each soil dilution was pipetted onto Mineral salt agar medium (MSA) to isolate H_2-oxidizing bacteria (Maimaiti et al. 2007; Schlegel and Meyer 1985) and incubated under the same condition for 2 weeks. Cycloheximide (10mg L^{-1}) was used to prevent fungal contamination. All bacterial colonies from the plates incubated were recovered and tested for their hydrogen oxidization ability using the Methylene Blue method (Lambert et al. 1985). One positive H_2-oxidizing bacteria strain *Variovorax paradoxus* (HQ689686.1) was used as positive control.

7.3.3 Identification of H_2-oxidizing bacteria

The bacterial colonies oxidizing Methylene Blue were identified using PCR (Polymerase Chain Reaction) – Sanger-sequencing method. Cells from the positive control strain and each positive isolate were picked up with a sterile needle and suspended into 50 µl TE buffer in a sterile 1.5 ml plastic centrifuge tube. Tubes filled with suspended cells were put into ultrasonic knapper (FS30H, Fisher Scientific, Pittsburg, PA, USA) to break bacterial cells. Crashed cell solutions were used as DNA template, and subjected to PCR using 16S rDNA-target primers 968f / 1401b amplifying

the universal bacterial gene. Platinum® PCR SuperMix (Cat. No. 11306-016, Invitrogen™) was used for PCR reactions. Thermal cycling was conducted in an Veriti™ 96-well fast Thermal Cycler (Applied Biosystems , California, USA) with the following conditions for the PCR: 4 min initial denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 56 °C, 1 min elongation at 72 °C; and 15 min of final elongation at 72 °C. Amplified bacterial DNA was purified by ChargeSwitch® PCR Clean-Up kit (Cat. No. CS12000, Invitrogen™). Purified DNA of each positive colony was sent for Sanger Sequencing at Plant Biotechnology Institute, Saskatoon, Canada. The sequences obtained were compared with sequences in the Genebank database using the BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/) and identified based on 97% sequence similarity.

7.3.4 Plant growth promoting effects of isolated H_2-oxidizing bacteria on durum wheat root elongation

The plant growth promotion ability of all five H_2-oxidizing bacterial isolates was tested on durum wheat (*Triticum turgidum* var *durum*Desf.) cultivar AC Avonlea seedlings following a protocol modified from Belimov (2001). The bacterial colonies were grown overnight on 30% nutrient broth (NB) medium at 28°C. Bacterial cells were then suspended into 50% sterile MSA solution without agar and yeast extract at the concentration of 5×10^7 cells per ml. Durum wheat seeds were surface sterilized with a mixture of 70% ethanol and 30% hydrogen peroxide (1:1) for 2 min and rinsed with sterile distilled water several times. Surface sterile seeds were pre-germinated overnight under 28°C in the dark. Seeds at the same germination stage were transferred into Petri dishes underlayed with filter paper. Five Petri dishes with 10 durum wheat seeds were prepared to test the growth promotion ability of each H_2-oxidizing bacterial isolate on

root elongation test. Six ml of bacteria suspension was added to each Petri dish. All dishes were covered and cultured at 28°C in the dark for 2 days. Sterile 50% MSA solution without agar and yeast extract was used as control. After the 2-day incubation period, the longest primary root length was measured from the node to the root tip.

7.3.5 Plant growth promoting effects of isolated H_2-oxidizing bacteria on chickpea growth

A greenhouse assay was used to test the effect of the H_2-oxidizing bacterial isolates with growth promotion ability, as identified *in vitro* on durum wheat, on the growth of chickpea (CDC Luna). Chickpea seeds were surface sterilized with the same chemicals as mentioned in section 7.3.4 for 2 min and rinsed with sterile distilled water. The three plant growth promoting bacterial isolates (L-3, L-6, L-11) were grown overnight on 30% nutrient broth (NB) medium under 28°C, and bacterial solutions were prepared as described above. Thirty surface sterile chickpea seeds were pre-germinated in each of the bacterial suspension for 2 days at room temperature, in the dark. Healthy germinated seeds were selected and transferred into pots filled with pasteurized field soil. Seeds pre-germinated with sterile MSA solution without bacterial isolates were used as control. Five pots were prepared for each bacterial isolate. Three seeds were sown in each pot, and plants were thinned to one per pot after emergence. Pots were grown in the greenhouse under day/night temperature of 22 °C / 15 °C and a photoperiod of 15h / 9 h, with 75 % relative humidity. Plants were given equal amounts of water as needed every two days. Pots were rotated every week to give all pots equal chances to be exposed to particular micro environmental conditions that may have existed on the greenhouse bench. After one month of growth, shoot height of the main plant stem was measured. All tested plants were removed from soil and wash off any loose soil and debris was

washed off with running tap water. Plants were dried in an oven (Therom Scientific, USA) set to 60 °C for two days, then plants were left to cool in a dry environment over night. Plants shoot and roots were weighed separately (OHAUS AV2101C Scale, NJ, USA).

7.3.6 Statistical analysis

DNA sequences were identified by comparison to known sequences using LBAST and Genebank (Table 7.1). Data collected from the root elongation and greenhouse bioassays were analyzed by ANOVA to test the significance of treatment effects, and Fisher-LSD test were performed to test the significance of differences among treatments means at $\alpha = 5\%$, using SYSTAT 12.0.

7.4 Results

7.4.1 Isolation and Identification of H_2-oxidizing bacteria

Different H_2-oxidizing bacterial isolates were identified among the 1286 bacterial cultures obtained from the 64 field soil samples through the Methylene Blue test (Figure 7.1). Results of the molecular identification of H_2-oxidizing bacterial isolates are presented in Table 7.1. *V. paradoxus* was reported as a H_2-oxidizing bacteria associated with soybean grown in a subhumid climate (Maimaiti et al. 2007). The H_2-oxidization abilities of the other three identified strains of *Rhodococcus* and *Sphingomonas* were not reported before.

7.4.2 The effect of H_2-oxidizing bacteria on durum wheat root elongation

Three of the five H_2-oxidizing bacteria isolates promoted durum wheat root elongation ($P < 0.0001$, Figure 7.2 and Table 7.2). Root length of durum wheat increased by 34.7 %, 64.5 % and 52.8 % after inoculation with *V. paradoxus* strain L-3, *R. erythropolis* strain L-6 and *V. paradoxus* strain L-11, respectively.

Table 7.1 Identity of the H_2-oxidizing bacteria isolated from chickpea rhizosphere, according to BLAST results.

Isolates	GenBank accession no. for closest match	Closest match from GenBank by BLAST
L-1	HQ864597.1	*Rhodococcus erythropolis* strain OR9 16S ribosomal RNA gene (100%)
L-3	HQ689690.1	*Variovorax paradoxus* strain IBP-SL9 16S ribosomal RNA gene (99%)
L-6	HQ864598.1	*Rhodococcus erythropolis* strain OR13 16S ribosomal RNA gene (100%)
L-11	AB627014.1	*Variovorax paradoxus* strain MKCM1007 16S ribosomal RNA gene (97%)
V-12	NR029327.1	*Sphingomonas asaccharolytica* strain Y-345 16S ribosomal RNA (99%)

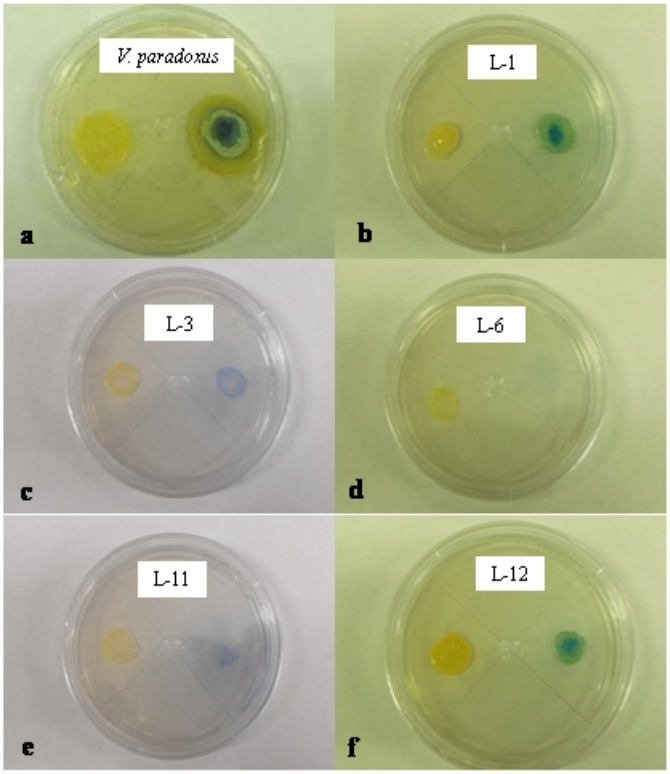

Figure 7.1 Five bacterial isolates from chickpea rhizosphere soil testing positive for H_2-oxidation in the Methylene blue assay. (a) H_2-oxidizing bacteria *V. paradoxus* strain as positive control. (b~f) positive isolates identified from this study as listed in Table 2. Two clones, with (right) and without (left) Methylene blue reagent, were grown on the same dish.

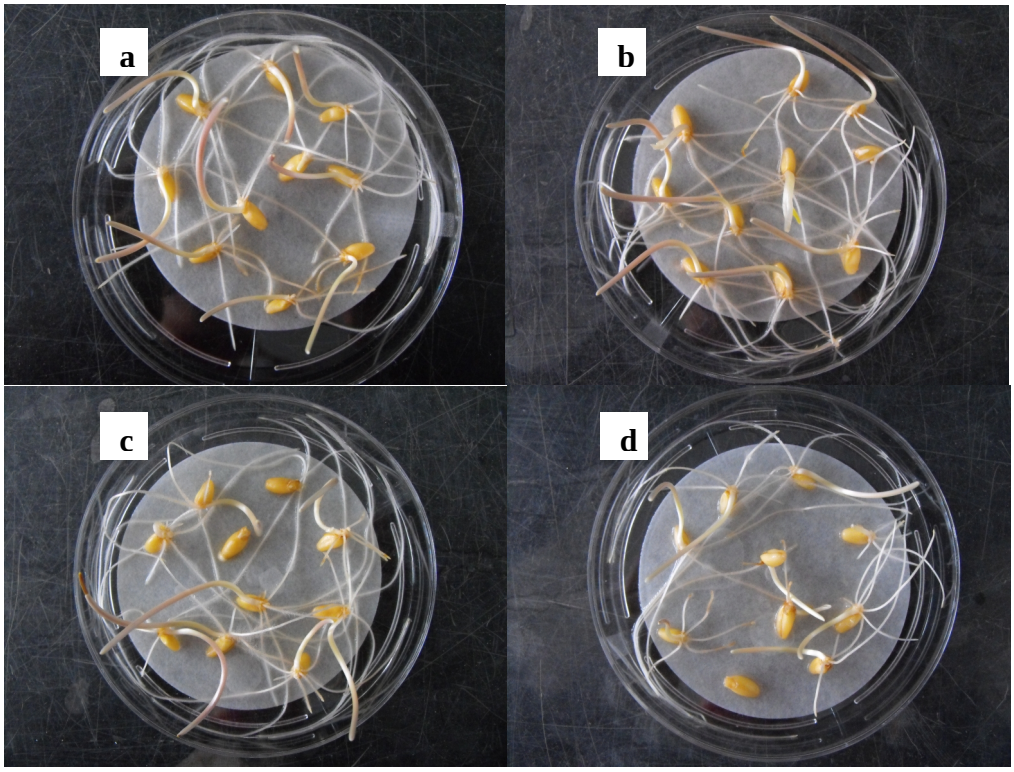

Figure 7.2 Growth promotion effects of H_2-oxidizing isolates on durum wheat growth. (a~c) Inoculated durum wheat seedlings with isolates L-3, L-6, L-11; (d) non-inoculated durum wheat seedlings (control).

Table 7.2 Effect of identified H_2-oxidizing bacterial isolates on the primary root elongation of durum wheat seedlings.

Isolates	Root elongation (cm)
L-1	4.62 c
L-3	5.2 b
L-6	6.35 a
L-11	5.9 ab
V-12	5.11 bc
Control	4.26 c
P value	< 0.0001

Note: Means associated with different letters are different at $P < 0.05$, $n = 5$.

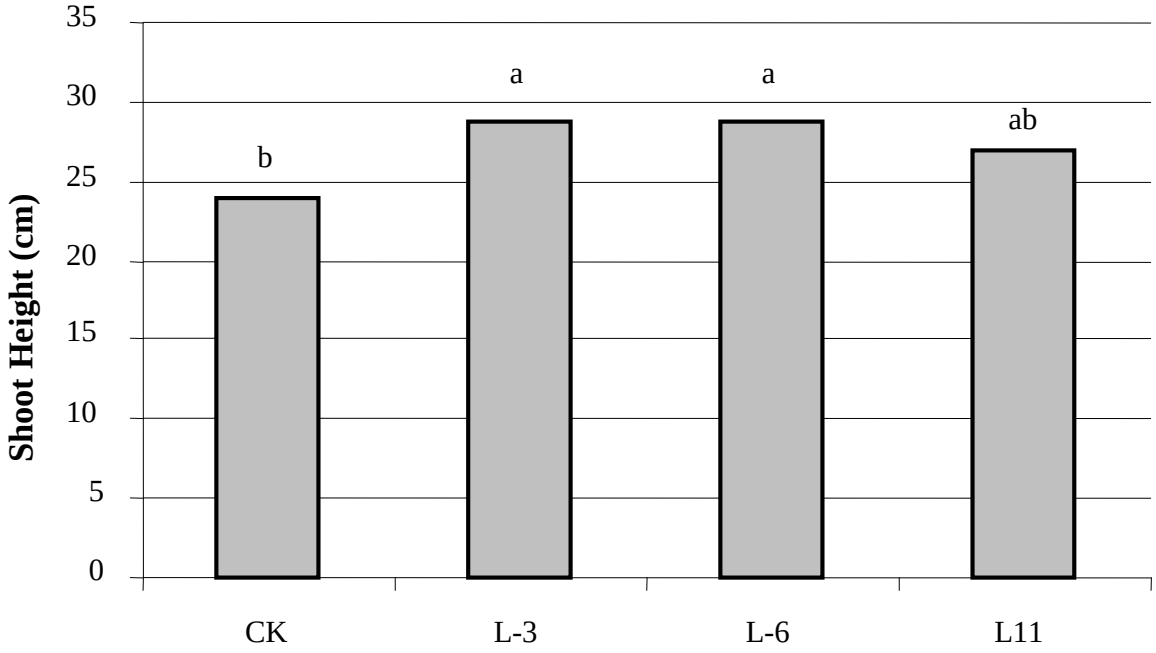

Figure 7.3 Chickpea plant height without and with inoculation of three H_2-oxidizing bacterial isolates (L-3, L-6 and L-11). Bars associated with different letters represent different means at $P < 0.05$; $n = 5$.

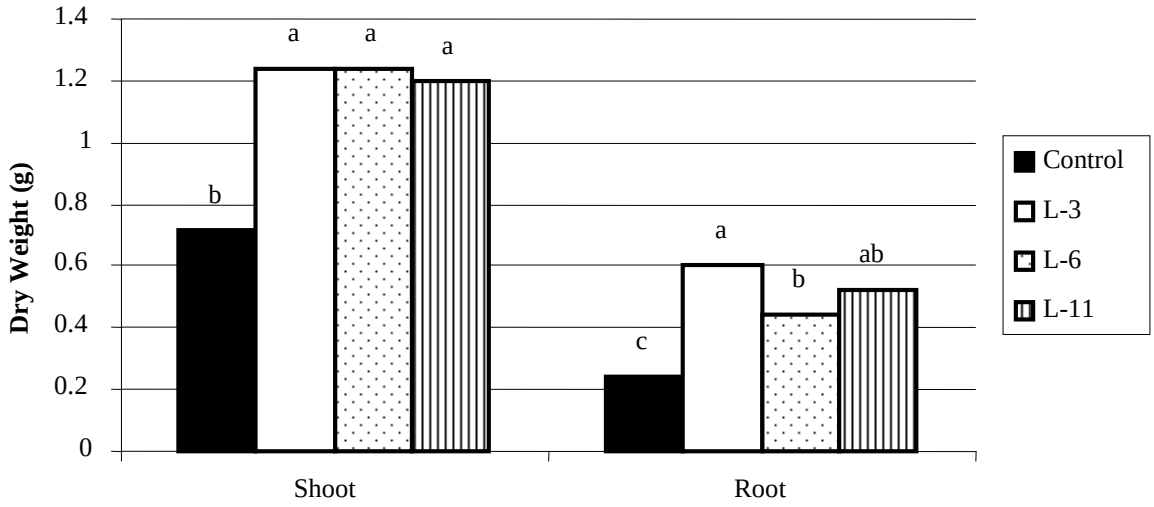

Figure 7.4 Chickpea plant shoot dry biomass and root dry mass after inoculation with the three H_2-oxidizing bacterial isolated (L-3, L-6 and L-11) or a sterile inoculant. Bars associated with different letters represent significant difference at $P < 0.05$ (ANOVA $P = 0.015$ for shoot and $P = 0.001$ for root dry mass; $n = 5$).

7.4.3 The effect of H_2-oxidizing bacteria isolates on chickpea growth

Chickpea plant height was increased by inoculation (P = 0.004, Figure 7.3). In particular, a 20 % increase in shoot height was seen in response to inoculation with *V. paradoxus* strain L-3, and a 19.7 % increase in response to inoculation with *R. erythropolis* strain L-6. Chickpea shoot dry mass was significantly increased by 72 % in response to inoculation with *V. paradoxus* strain L-3, increased by 72.2 % in response to inoculation with *R. erythropolis* strain L-6, and increased by 66.7 % in response to inoculation with *Variovorax paradoxus* strain L-11 (P = 0.015, Figure 7.4). Chickpea root dry mass was similarly increased after inoculation with these three bacteria isolates. Particularly, chickpea root dry weight increased 150 % in response to inoculation with *V. paradoxus* strain L-3, increased by 83.3 % in response to inoculation with *R. erythropolis* strain L-6, and increased by 116.7 % in response to inoculation with *Variovorax paradoxus* strain L-11 (P = 0.001, Figure 7.4).

7.5 Discussion

This study demonstrates the existence of H_2-oxidizing bacteria in chickpea rhizosphere soil, and in semiarid environment. Pulse crops have a particular influence on soil microorganisms. Hydrogen gas as the by-product of nitrogenase activity diffuses in soil from legume root nodules. This hydrogen is used as an energy source by H_2-oxidizing bacteria living in the vicinity of the root nodules (La Favre and Focht 1983). The previous research on H_2-oxidizing bacteria associated with legumes is limited to soybean (Dong et al. 2003; Maimaiti et al. 2007). This study presents the first report of H_2-oxidizing bacteria in chickpea rhizosphere. *Variovorax* spp. (Betaproteobacteria) were reported for their H_2-oxidizing activity and plant growth promotion ability in soybean rhizosphere (Maimaiti et al. 2007), but the H_2-oxidizing and plant growth promoting

capabilities of Actinobacteria (*R. erythropolis*) and the Alphaproteobacteria (*S. asaccharolytica*) are reported for the first time. H_2-oxidizing bacteria belong to different bacterial phyla including Aguificae, Gammaproteobacteria and Betaproteobacteria (Hayashi 1999). The present results indicate that the H_2-oxidation metabolism may be more widely distributed in the soil microbiota than previously thought.

In this study, I found growth-promoting effects of H_2-oxidizing bacterial isolates on durum wheat root elongation. Maimaiti (2007) found that H_2-oxidizing bacteria strains such as *V. paradoxus, F. johnsoniae* and *B. sordidicola* promoted plant growth of cereal crops. In crop rotation sequences, cereal crops usually produce higher yields after pulses (Ryan et al. 2010). Increased soil N fertility was believed to be the reason explaining better cereal crop growth following legume crops (Pierce and Rice 1988). Research found that enhanced cereal crop yield was also related to the activity of bacteria supported by hydrogen gas metabolism (Irvine et al. 2004). Some H_2-oxidizing bacteria not only have the ability to metabolize hydrogen gas, but they also promote plant growth. Three of the five H_2-oxidizing bacterial isolates encountered in the chickpea fields had plant growth promoting activity in this study, which may be due to phytohormones they secreted, as Belimov (2001) found that *Variovoras paradoxus* can produce ACC deaminase and rhizobitoxine. Rhizobitoxine can promote nodulation of legumes by inhibiting ethylene synthesis (Okazaki et al. 2007), while ACC deaminase can reduce the production of ethylene and sustain plant growth under stressful conditions, such as drought, salinity, and pathogen affection (Saleem et al. 2007). As the consequence, these bacteria can promote plant growth.

Most research on the growth-promoting effects of legumes selected H_2-oxidizing bacteria focused on the crops following the soybean (Maimaiti et al. 2007), and few of

them reported growth-promoting effects of these bacteria on the legume crops (Dong et al. 2003). The present study confirms the plant growth promoting effects of some H_2-oxidizing bacteria also on the pulse plants.

7.6 Conclusion

Biological nitrogen fixation is such an important functionality of soil bacteria which certainly yield benefits to plants. Hydrogen gas, as a by-product of biological nitrogen fixation, may influence plant performance by impacting specific soil bacteria group which has plant growth promoting ability. I found several H_2-oxidizing bacteria strains in chickpea field in semi-arid area, which can increase chickpea growth. These results showed that the distribution of H_2-oxidizing bacteria in the soil is wider than previously thought, and presence of these bacteria in chickpea field have the potential benefits on both chickpea and the durum wheat crops in pulse-cereal rotation.

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8. Preface

The following section presents the general conclusion based on all data. It reveals effects of fungicide inputs and chickpea genotypes on rhizobacterial communities in chickpea field. Different fungicide application strategies exhibited diverse rhizobacterial communities, which may because various feedback metabolisms of these bacteria to different fungicides. Meanwhile, varied pulse crops affected their rhizobacterial communities differently, due to their respective growth habits. Weather conditions, such as precipitation, also influenced rhizobacterial community.

8. GENERAL DISCUSSION

Soil microorganisms carry on many important functions in soil and plants, such as biological nitrogen fixation, nutrient cycling, soil organic matter turnover, soil humus formation, and soil physical structure building. These functions are performed by many different genera and species of microorganisms, and soil microbial diversity is critical to soil functioning. The composition and diversity of soil microbial communities is often influenced by crop management. My research assessed the effect of fungicide applications and crop genotypes on the soil bacterial community and on plant-bacteria interactions in chickpea fields.

8.1 Fungicide effects

In this study, I detected sizeable amounts of fungicide residues in rhizosphere soil samples collected from fungicide treated chickpea field. Although foliar fungicide application is commonly applied in chickpea production worldwide as the crop is very susceptible to fungal pathogens (Chang et al. 2007; Demirci et al. 2003; Wise et al. 2008), the effects of fungicides on other microorganisms of the agroecosystems are not well documented. Several fungicide strategies applied in my research did not result in noticeable richness changes in the general bacterial community, but the composition of the rhizobacterial community was significantly changed compared to untreated plots. The application of fungicide may inhibit the growth of particular bacterial groups while stimulating others, as different soil bacterial groups have different metabolisms (Huang et al. 2010).

In particular, fungicide application changed the structure of the N_2-fixing bacterial community. Nodulation was directly impacted by fungicide use. Fungicide application reduced nodule size, which concurs with former research (Petit et al. 2008). However, plants receiving more chemicals had smaller nodules, but fixed more N_2 than non-treated plants, indicating that nodule size does not reflect reduced N_2-fixing activity in fungicide treated plants. According to previous research, nodulation is controlled by specific chemical signalling (Geurts et al. 2005), and changes in plants root secretions due to fungicide influences may explain the changes observed in the N_2-fixing bacterial community in chickpea rhizosphere.

8.2 Chickpea genotype effects

I found that different chickpea genotypes selected different rhizobacterial communities. Based on previous research, general rhizobacterial diversity and density are restricted by the availability of organic C, such as root secretions, sloughing-off of root cap cells, and senescencing root epidermis (Nguyen 2003), which varied with plant genotypes. Besides, crop genotype effects on their associated rhizobacterial communities may also due to their different growth habit, such as termination time. The indeterminate growth habit of chickpea plants means that the bacterial succession taking place in the rhizosphere can span over a longer period, and shape the composition of rhizobacterial communities in a more specific way among different chickpea genotypes. Contrasting soil bacterial communities can be observed at different crop growth stages (Andreote et al. 2010). The influence of plant growth stage on their associated microbiota was attributed to changes in plant secretions (Meier et al. 2008). For example, young roots provide more energy and nutrient sources, stimulating the growth of r-strategist bacteria (Bowen and Rovira 1991; Lynch and Whipps 1990), while old roots promote the relative

abundance of K-strategist, which require less energy and nutrient than r-strategist (Andrews and Harris 1986; Zhang et al. 2011).

Composition of some functional bacteria groups, such as N_2-fixing bacteria, also varied with chickpea cultivars. The symbiosis between N_2-fixing bacteria and their host plants is controlled by specific symbiotic signalling chemicals called flavonoid (Geurts et al. 2005) produced by pulse roots in amount and quality that differ with the pulse species and cultivar. Therefore, differences in the signalling system of different chickpea genotypes could results in differences in the nodulation pattern between the plants or in the structure of the N_2-fixing bacterial communities in their rhizosphere.

8.3 Potential effects of bacterial community at pulse stage, on the growth of subsequent crops

Pulse crops bring many benefits to cropping system, such as increased soil available N (Lindström et al. 2010), enhanced soil water content (Miller et al. 2003), and reduced cereal disease outbreaks (Stevenson and Van Kessel 1996b). In this study, I found that the selective effects of different pulse crops on plant-growth-promoting organisms may explain the benefits of pulses in cropping systems. Previous research showed that plant growth promoting rhizobacteria can reduce disease pressure and produce growth stimulating compounds (Hayat et al. 2010). Several bacteria from the phyla that were dominant in my study, in particular Proteobacteria and Actinobacteria, were reported for their growth promoting effects on wheat (Hamdali et al. 2008; Jha and Kumar 2009; Nabti et al. 2010). Greenhouse bioassays testing the effects of different pulse rhizosphere microbial communities on durum wheat also showed that the organisms living in pulse crop rhizosphere have different growth-promotion potential, which may differently affect the growth of a subsequent crop.

Changes in the endophytic bacterial community structure of durum wheat roots, induced by a previous pulse crop, were related with durum wheat yield, in the field. Previous study reported that cereal plants grown after pulse in different pulse-cereal rotations associated with different rhizobacterial communities (Alvey et al. 2003), indicating that the former pulse crops impact their following cereal crops indirectly, through changes in the soil bacterial community. In this study, durum wheat grown after chickpea had different bacterial endophyte community and yield, than durum wheat grown after yellow pea. This effect was mainly due to the different termination time of these two pulse crops. In wheat, high grain yield was correlated with abundant colonization of roots by Proteobacteria and Actinobacteria, here and elsewhere (Conn and Franco 2004; Iniguez et al. 2004). Bacteria of these phyla were the dominant colonizers of durum wheat following an early terminated pulse crop. By constrast, Firmicutes which dominated the rhizosphere of chickpea at late growth stage (Wu et al. 2010), were negatively correlated with durum wheat productivity. Therefore, the effects of a former chickpea crops on the bacterial endophyte of a following durum wheat crop, in cropping systems seemingly explain why chickpea is generally less beneficial to the following cereal crops than pea.

8.4 Conclusion

This Ph.D. thesis reports on how chickpea rhizobacterial communities are influenced by agronomic decision, and explains an important part of the rotation effect of chickpea on a following durum wheat crop in pulse-cereal rotation. Intensive fungicide application on chickpea plants induces changes in soil bacteria, and this shift may affect the ecological functions of the soil. Chickpea genotypes had an impact on the composition of their associated rhizobacterial communities, which may further influence

the activities of functional bacterial groups, and the growth of following crops in cropping systems. Therefore, agronomic decisions considering the influence of fungicide application strategies and crop cultivars on rhizosphere organisms can optimize the ecological services of soil microorganisms to crop production.

For the further research, effects of chickpea genotypes and agronomic practices on chickpea associated functional bacterial subgroups will be studied. Such as the N_2-fixing bacteria I identified from chickpea field. Although neither fungicide nor genotype effects on H_2-oxidizing bacterial group was detected (data not shown), the identification of this specific plant-growth-promoting bacterial group give us an chance to review the existence of these functional bacteria and their influence on host plant growth in chickpea field, and identification of these functional bacteria from the chickpea rhizosphere can provide us an opportunity to optimize the biological functions of the plant-soil eco-system, which can lead to better production of pulse-cereal rotation and increased benefits for crop producers.

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